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Differential Toxicity of Vincristine and Vinblastine Against Human Promyelocytic Leukemia HL-60/C1 Cells

by

Peter J. Ferguson

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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Dedication

To Susan

Abstract

Vincristine (VCR) and vinblastine (VLB) differ in the spectra of their anti-tumor activities and in their dose-limiting toxicities. A clonal line of human promyelocytic leukemia cells, HL-60/C1, was tested in culture for sensitivity to VCR and VLB during continuous and short-term exposures. The concentrations of VCR and VLB, respectively, that inhibited growth rates by 50% (IC_{50} values) during 48-hr continuous exposures were 7.6 and 8.1 nM. When cells were subjected to 4-hr exposures and transferred to drug-free medium, IC_{50} values for inhibition of proliferation by VCR and VLB, respectively, were 41 nM and 1.1 μM and for inhibition of colony-formation were 21 nM and 3.7 μM. Thus, although VCR and VLB were equitoxic during continuous exposures, VCR was more toxic than VLB when cells were subjected to exposures of limited duration and then transferred to drug-free medium. Measurements of DNA distributions of VCR and VLB-treated cells (continuous exposures) by flow cytometry demonstrated that growth-inhibited cells accumulated in the G_2-M phases of the cell cycle to the same extent, suggesting the same cytotoxic mechanism for both drugs. Cellular uptake and release of [^{3}H]VCR and [^{3}H]VLB were examined under conditions of growth experiments. During continuous exposures, VLB uptake was more rapid than that of VCR and reached maximal values much earlier. The relationships between cell viability and "effective drug exposures" (determined by calculating *areas under curves* of plots of *cellular drug content versus time*) were the same for VCR and VLB. After 4-hr exposures to a given concentration of drug, the cellular content of VLB was greater than that of VCR, and when cells were transferred to drug-free medium, VLB was released more rapidly and to a greater extent from cells than was VCR. Loss of cell viability occurred at lower "effective drug exposures" of VCR than of VLB. Rates of uptake and release of VLB by HL-60/C1 cells were unaffected by depletion of cellular ATP by azide poisoning and glucose starvation, indicating that uptake and

release of VLB were not dependent on energy metabolism. [^{3}H]VCR and [^{3}H]VLB bound to serum proteins in growth medium to the same extent (25%) over the wide range of concentrations used in cytotoxicity studies. At lower serum concentrations, less drug was bound to proteins, but more was taken up by cells, suggesting that free, and not protein-bound drug entered cells. Cellular drug content following 4-hr exposures increased with increasing pH (from 5.5 to 7.5), suggesting that since VLB is more lipophilic than VCR, the greater uptake of VLB than VCR may have been due to hydrophobic interactions.

In summary, differential toxicity was attributed to the more rapid loss of VLB than VCR when cells were subjected to short exposures to these agents, followed by culture in drug-free medium. The differences in cellular uptake and release of VLB and VCR by HL-60/C1 cells appeared to be related to the greater hydrophobicity of VLB rather than to differences in mediated uptake or efflux of the two drugs. VLB, which is considerably more hydrophobic than VCR, should diffuse across the plasma membrane more readily and should bind nonspecifically to hydrophobic cellular components to a greater extent than VCR.

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List of Abbreviations

ACT-D	actinomycin D
ADR	adriamycin
ATP	adenosine triphosphate
CH	Chinese hamster cells
CLC	colchicine
cpm	counts per minute
CTP	cytidine triphosphate
CYTO-B	cytochalasin B
Dem	demecolcine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNR	daunorubicin
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
FBS	fetal bovine serum
GTP	guanidine triphosphate
Hepes	N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
HMBA	hydroxymethylbutyric acid
HPLC	high performance liquid chromatography
IC_{50}	concentration that inhibits proliferation or colony-formation by 50%
MAYT	maytansine
MITHRA	mithramycin
MITO-C	mitomycin C
mono	monocyte
MTX	methotrexate

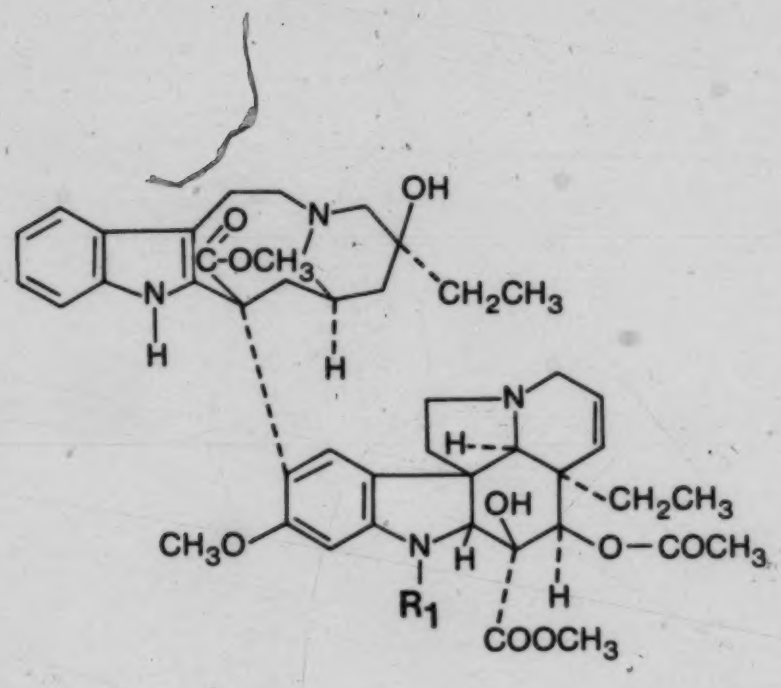
NBT	nitroblue tetrazolium
PMN	polymorphonucleocyte
PURO	puromycin
ROS	Ridgway osteogenic sarcoma
RPMI 1640	Roswell Park Memorial Institute medium 1640
S.D.	standard deviation
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	tris (hydroxymethyl) aminomethane
VCR	vincristine
VDS	vindesine
VLB	vinblastine
VM-26	teniposide
VP-16	etoposide
XTP	xanthine triphosphate

I. Introduction

Vincristine (VCR) and vinblastine (VLB), two anticancer agents used clinically since the early sixties, are the most active isolates of the periwinkle plant, *Catharanthus roseus* G. Don. (1). VCR and VLB are similar in molecular structure (Figure 1). They consist of the same indole-indoline heterocycle of molecular weight 795 (2) and differ by one side group, which for VLB is a methyl group and for VCR is a formyl group (3). Both drugs are "antimitotic agents", inhibiting mitosis and other cellular processes dependent on microtubules by binding to tubulin, the protein subunit of microtubules.

Despite similarities in structure and function, VCR and VLB are used clinically in the treatment of different tumor types (4). VLB is used, in combination with other agents, to treat breast carcinoma, choriocarcinoma, Hodgkin's disease, non-Hodgkin's lymphomas, and testicular carcinoma. VCR is used to treat acute lymphocytic leukemia, breast carcinoma, Ewing's sarcoma, Hodgkin's and non-Hodgkin's lymphomas, neuroblastoma, rhabdomyosarcoma, and Wilm's tumor. Because VLB is less toxic than VCR, it is administered in larger doses, 3.7-7.4 mg/m^2 i.v. weekly or every two weeks, compared to 1.0-2.0 mg/m^2 i.v. weekly for VCR, with doses of the latter not exceeding 2.0 mg (4).

The dose-limiting toxicity of VLB is leukopenia. VLB also causes stomatitis, constipation or diarrhea, and nausea and vomiting, readily controlled by antiemetics. Neurologic complications, alopecia, thrombocytopenia, and anemia are uncommon. The most severe side effects of VCR are on the nervous system. Although sensory impairment and parasthesias are common, but not dose-limiting, loss of deep tendon reflexes, ataxia, and muscle wasting are dose-limiting. VCR causes alopecia in about 20% of patients, and toxicity towards hematopoietic cells has been demonstrated in normal use of VCR (5) and in patients inadvertently given overdosages of VCR (6, 7). The selective neurotoxicity of VCR limits allowable doses to about one-fourth



	R_1
Vinblastine	CH_3
Vincristine	CHO

Figure 1. Molecular structure of VCR and VLB. (from reference 36.)

those of VLB. The basis of the differential clinical activity of VCR and VLB is unknown. Results from pharmacokinetic studies suggest that selective retention of VCR by some tissues may contribute to its greater toxicity against the host. Most (>99%) of the total dose of either drug is cleared from the serum of patients within 4 hr following a bolus injection (8, 9, 10, 11, 12). The rapid clearance from serum is due to distribution of VCR or VLB in body tissues within minutes and excretion via the hepato-biliary route. VCR is retained in the body for longer periods than VLB, suggesting that there may be differences in tissue uptake[1] and release of VCR and VLB.

This project was undertaken to characterize differential activity[2] of VCR and VLB at the cellular level and to determine if differential activity is related to differences in uptake (or release) of VCR and VLB. Results of preliminary experiments that were undertaken to identify cell types that exhibit differential sensitivity to the *Vinca* alkaloids have been reported (13) and are presented in summary form in Table 1. Tumor cell lines in culture were used as model systems, and two of the lines studied, mouse neuroblastoma and human promyelocytic leukemia HL-60, were included because it was thought that they might exhibit greater sensitivity to VCR and VLB, thereby reflecting the selective toxicity of these drugs seen *in vivo* against nervous and hematopoietic tissue.

Differential activity of VCR and VLB was seen only after relatively short exposures to drug (13). When cells were exposed to either drug continuously over a period of several generation times, VLB and VCR were equitoxic against 5 of the 6 cultured cell lines tested. After long exposures (24-96 hr, depending on the cell type), there were no differences in the antiproliferative activities of VCR and VLB

---

[1] "Uptake" includes passage of drug across the plasma membrane and its subsequent interaction with cellular components.
[2] The term "differential activity" refers to the differences between the biological actions of VCR and VLB with respect to toxicology and therapeutic effects in experimental systems and clinical use.

[1]For proliferation experiments, the period during which cell numbers were determined for calculation of proliferation rates. For assay of colony-formation, cultures were incubated at 37° for 10-14 days, and the number of colonies was scored.

[2]uncloned parent line originally obtained from Gallo *et al.* (14).

To determine the effects of VCR and VLB on proliferation, cells were exposed continuously for the lengths of time indicated or exposed for 4 hr and cultured for the times indicated in the appropriate drug-free growth medium. IC_{50} values are the concentrations of drug that inhibited proliferation rates, relative to those of untreated cultures, by 50% over the period of evaluation. To determine the effects of VCR and VLB on viability, cells were exposed for the times indicated, and colony formation was assayed in the absence of drug on culture plates (HeLa) or in soft agar (L1210). IC_{50} values are the concentrations of drug that reduced colony formation, relative to that of untreated cultures, by 50%. The number of experiments used to calculate IC_{50} values are indicated in parentheses, and, for most values, the mean (± S.D.) is presented. (From Ferguson *et al.*, Cancer Research, 44: 3301-3312, 1984, ref. 13.)

Table 1

IC_{50} Values for Inhibition of Proliferation and Colony-Formation of Cultured Cells by VCR and VLB

Cell Line	Length of Exposure (hr)	Length of Evaluation Period[1] (hr)	IC_{50} (nM) for Proliferation	
			Vincristine	Vinblastine
HeLa	48	48	1.4±0.6(2)	2.6(1)
S-49	48	48	5(1)	3.5(1)
HL-60[2]	96	96	4.1±0.7(4)	5.3±0.4(3)
	4	72	23±6(3)	900±100(3)
L1210	48	48	4.4(1)	4.0(1)
	4	48	100±50(3)	380±140(6)
Neuroblastoma	48	48	33±3(2)	15±1(3)
	4	48	250±100(4)	290±40(3)
			IC_{50} (nM) for Colony-Formation	
HeLa	24		1.2(1)	<3(1)
	1		33±8(3)	62±4(2)
L1210	24		5.4(1)	2.5(1)
	4		6.0±0.7(2)	600-37,500(5)

against mouse lymphoma S49, mouse leukemia P388, mouse leukemia L1210, human cervical carcinoma HeLa or human promyelocytic leukemia HL-60 cells, and VLB was only slightly more active than VCR against mouse neuroblastoma cells. In contrast, when cells were exposed to either drug for 4 hr,[1] followed by culture in drug-free medium, VCR was considerably more toxic than VLB against mouse leukemia L1210 and human promyelocytic leukemia HL-60 cells, and, when exposures of 1 hr were assessed, VCR was 2-fold more toxic than VLB against HeLa cells. Differential toxicity was not seen, after short exposures, against mouse neuroblastoma cells. Since the greatest difference in activity of the two drugs was seen with HL-60 cells, they were employed for further study.

HL-60 cells were established in culture from a female patient with acute promyelocytic leukemia (14). HL-60 cells have the potential to differentiate towards granulocytic cells or macrophage/monocytic cells when induced by different chemicals (18, 19). When HL-60 cells were introduced into this laboratory, the population doubling time of logarithmically proliferating cultures was 36-48 hr, and the cloning efficiency was 10% (20). An improved cloning method was developed, and a subline, HL-60/C1, was established from a single colony. Exponentially proliferating cultures of HL-60/C1 cells had a population doubling time of 24 hr and a cloning efficiency of 50%. HL-60/C1 cells retained the capacity to differentiate when treated with the appropriate chemicals.[2]

The biological effects of VCR and VLB were characterized using the clonal derivative, HL-60/C1. Under conditions of continuous exposure for 48 hr, VCR and VLB exhibited the same activity against HL-60/C1 cells, inhibiting proliferation rates by 50% (IC_{50} value) at 7.6 and 8.1 nM, respectively. In contrast, when HL-60/C1 cells were exposed to drug for 4 hr, and then cultured in drug-free medium for 48

---

[1]Four-hr exposures were used because VCR and VLB are almost totally cleared from serum of humans within 4 hr of a bolus injection (11, 15, 16, 17).
[2]Ferguson, P. J., unpublished results.

hr, VCR was more toxic than VLB. The IC_{50} values for inhibition by VCR and VLB of proliferation rates differed by 27-fold and for colony-formation by 180-fold.

Inhibition of mitosis by perturbing mitotic spindle formation has been correlated with cytotoxicity of VCR and VLB against cultured cells (21, 22, 23, 24). Since VCR and VLB have been shown *in vitro* to bind with nearly equal affinity to free tubulin (25) and to inhibit polymerization of microtubules with nearly equal efficiency (26), experiments were undertaken to determine if VCR and VLB exerted their cytotoxicity against HL-60/C1 cells through the same target. Inhibition of mitosis was evaluated by monitoring DNA distributions of drug-treated cells by flow cytometry. Comparison of DNA distributions in cells treated for short (4-hr) or long (24-hr) intervals suggested that VCR and VLB inhibited proliferation by the same mode of action. For both types of exposures, the DNA distributions obtained from cells treated with equitoxic levels of VCR or VLB were virtually identical. During continuous exposures, both drugs caused accumulation and arrest of cells in the G_2-M phases of the cell cycle in a concentration-dependent manner, suggesting that inhibition of mitosis was probably the biological mechanism of cytotoxicity.

VLB is taken up and released by platelets (27) and L1210 cells (13) more readily than VCR. If L1210 cells are exposed to similar concentrations of [^{3}H]VCR and [^{3}H]VLB, VLB enters cells more rapidly than VCR, and if cells are "loaded" with the same amount of [^{3}H]VCR or [^{3}H]VLB, VLB is released more rapidly than VCR (13). Thus, it was possible that differential uptake and/or release of VCR and VLB by HL-60/C1 cells may have given rise to the differential toxicity seen after short exposures.

To assess the role of uptake and release in differential toxicity of the Vinca alkaloids against HL-60/C1 cells, the cellular content of [^{3}H]VCR and [^{3}H]VLB was determined during drug exposures that were the same as those used in evaluation of anti-proliferative activity. During continuous exposures, the amount of VCR in cells

at 24 hr was the same as VLB at all concentrations tested. Although VLB entered HL-60/Cl cells more rapidly than VCR during during the first few hr of exposure, when "effective drug exposures" were quantitated by measuring *areas under curves* of *cellular drug content versus time* from time 0 to 48 hr, VCR and VLB were equitoxic to HL-60/Cl cells. Thus, although there were differences in rates of uptake of VLB and VCR, the pharmacological effects of the 2 drugs on HL-60/Cl cells during continuous exposures were identical.

After 4-hr exposures, the amount of [^{3}H]VLB in cells was greater than that of [^{3}H]VCR at all concentrations tested. The cellular content of either drug at 4 hr was concentration-dependent, and the difference between cellular contents of [^{3}H]VCR and [^{3}H]VLB was greater at higher concentrations. When cells "loaded" with the same amount of radiolabelled drug were transferred to drug-free growth medium, the decrease in intracellular content of VLB was much faster than that of VCR. Effective drug exposures were quantitated by calculating the *areas under curves* of *cellular drug content versus time* during drug exposure, followed by culture in drug-free medium. In contrast to the results obtained for continuous exposure, VCR was much more toxic than VLB. These results suggested, since the cellular content of VLB was greater than that of VCR, that much of the VLB associated with cells during 4-hr exposures was not bound to the target of cytotoxicity.

Several other aspects of drug uptake were also examined. Both VCR and VLB are metabolized to varying degrees in liver (28, 29, 30, 31, 32, 33) and plasma (10, 34) of humans and rats. [^{3}H]VLB does not appear to be degraded by cell culture medium or by CCRF-CEM leukemic lymphoblasts (35). Since differences in metabolism of VCR and VLB by HL-60/Cl cells could have contributed to differential toxicity, ethanol extracts of cells and medium exposed for 24 hr to [^{3}H]VCR or [^{3}H]VLB were analyzed by high performance liquid chromatography (HPLC) for possible metabolites. Neither VCR nor VLB were significantly

metabolized by HL-60/C1 cells or degraded by enzymes present in the serum component of the growth medium.

Since VCR and VLB bind to proteins present in human serum (36, 37), binding of [^{3}H]VCR and [^{3}H]VLB to proteins of fetal bovine serum in growth medium was analyzed by equilibrium dialysis. At pH 7.5, both VCR and VLB were bound to the same extent by proteins present in fetal bovine serum, indicating that differences in extracellular concentrations of "free" drug did not contribute to differential toxicity. The effect of pH on binding to proteins of fetal bovine serum and on cellular uptake was also studied since VCR and VLB are alkaloid in nature, each with two pKa values (5-5.4 and 7.4) (38). At pH values <6, the majority of VCR and VLB molecules are ionized. However, despite their similar pKa values, the pH profiles obtained for VCR and VLB for serum binding and cellular uptake were markedly different. As pH values were raised from 5.5 to 8.0, binding of VLB to serum proteins increased 2-fold, whereas that of VCR was relatively unchanged. For both drugs, cellular uptake increased as pH values were raised, and at pH values >7.5, when VCR and VLB were relatively uncharged, the amount of cell-associated VLB was much greater than that of VCR. The greater hydrophobicity of VLB, relative to VCR, may be responsible for the greater sensitivity of serum protein binding and cellular uptake to pH.

It has been suggested that the *Vinca* alkaloids may enter cells by mediated transport (39, 40), although the evidence may be interpreted otherwise. Evidence has also been presented for the existence of an energy-dependent efflux system in drug-resistant cells (39, 41, 42, 43). The latter suggestion is based on observations in several cell-drug systems, including the *Vinca* alkaloids, that release of drug from cells is inhibited by depletion of cellular ATP.[1] An alternate interpretation is that

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[1]Energy-dependent release of drug has also been observed for adriamycin and daunorubicin in cells resistant to these drugs and to *Vinca* alkaloids (41, 42).

energy-dependent inhibition of binding of drug to cellular components is responsible for rapid release of drug (35). Uptake and release of [^{3}H]VLB was investigated in energy-depleted HL-60/C1 cells to determine if the putative energy-dependent efflux system was somehow involved in differential toxicity. Because rates of uptake and release of VLB by HL-60/C1 cells were higher than those of VCR, VLB was studied first, and in light of the results, VCR was not studied in energy-depleted cells. Uptake of [^{3}H]VLB during 4 hr and the rate of release into drug-free medium were not affected by treatment of HL-60/C1 cells with sodium azide under conditions that reduced cellular ATP below levels of detection. Thus, the rapid release of VLB was not mediated by an energy-dependent efflux system.

II. The Vinca Alkaloids

A. Introduction

Crude extracts of stems, leaves, and roots of the periwinkle plant, *Catharanthus roseus* G. Don (formerly *Vinca rosea* Linn), were commonly administered as a remedy for hypoglycemia during the early part of the twentieth century by doctors in the West Indies and England. Although people diagnosed as having diabetes were given such treatment, outside observers could ascertain no efficacy in treatment with periwinkle extracts (2). In 1949, Robert L. Noble, Charles T. Beer, and J. Harry Cutts, at the Department of Medical Research, University of Western Ontario, London, treated normal and diabetic rats and rabbits with periwinkle extracts, but found no evidence of anti-diabetic activity (2). The hematopoietic toxicities observed in these studies suggested that extracts might contain antiproliferative agents, and carcinostatic activity was subsequently demonstrated against a transplantable mammary adenocarcinoma in mice and a transplantable sarcoma in rats. Johnson *et al.* (44) at the Lilly Research Laboratories in Indianapolis also demonstrated antineoplastic activity in fractions isolated from crude *Vinca* extracts and subsequently found that mice bearing an ascites leukemia survived 90-120 days (cured) when treated with VLB. By 1959, VLB had reached clinical trials in cancer patients (45), although little was known about its cytotoxic mechanisms.

The action of VLB as an antimitotic agent was first established by Palmer *et al.* (46) and Cutts (47), and Cardinali *et al.* (48) later showed that VCR also inhibited mitosis. In 1964, Creasey and Markiw (49) found that treatment of mice bearing the Ehrlich ascites carcinoma with VCR or VLB inhibited protein synthesis and production of soluble, but not ribosomal, RNA in tumor cells. An increase in the proportion of tumor cells in mitosis was observed, and these authors speculated

that inhibition of synthesis of certain proteins was the basis of mitotic inhibition. In cultured Sarcoma 180 cells, the *Vinca* alkaloids, at concentrations that inhibited proliferation, had no effect on respiration, glycolysis, nucleic acid synthesis, or protein synthesis (50).

Chemical identification of the active substances in *Vinca* extracts was reported in 1955. The pH-dependent solubility of the isolates indicated them to be of an alkaline nature, hence the name "*Vinca* alkaloids" (2, 51, 52). VLB, or vincaleukoblastine, as it was then known, was the first alkaloid to be isolated and characterized as a single moiety (2, 53). It was found to be a heterodimer of two other isolates of the extract, catharanthine and vindoline. The latter compounds are believed to be metabolic derivatives of tryptophan (54). VCR was characterized later (3) and differs from VLB in having a formyl, rather than a methyl, group bound to the nitrogen in position 1 of the vindoline moiety (see Figure 1, Chapter 1). The molecular formulae of VCR and VLB, respectively, are $C_{46}H_{54}N_4O_{10}$ (molecular weight, 824) and $C_{46}H_{56}N_4O_9$ (molecular weight, 810) (2). The two basic nitrogens of the *Vinca* alkaloids have similar pKa values (38). A pKa of 7.4 is common to both VCR and VLB, and the second pKa values are, for VCR and VLB, respectively, 5.0 and 5.4. The free bases are soluble in ethanol and insoluble in water, and pharmaceutical preparations are generally available as sulfate salts. VLB has absorbance maxima at 214 and 259 nm (51) and VCR at 220, 255, and 296 nm (55). The molar extinction coefficient for both VCR (255 nm) and VLB (259 nm) is 16,218 cm^{-1} M^{-1}.

B. Antimitotic Activity of the Vinca Alkaloids

Numerous studies with cultured cells have established that the *Vinca* alkaloids inhibit mitosis in proliferating cells. There was a correlation between the rate of loss of colony-forming ability of mouse L-cells and the rate of accumulation of mitotic

cells upon exposure to VLB (21). Two strains of Chinese hamster fibroblasts that differed 100-fold in their sensitivity to VLB each exhibited similar logarithmic relationships between drug concentration and (a) dissolution of the mitotic apparatus and (b) cell kill (16). Earle's L-cells, after exposure to 10 nM VLB for 21 hr, were arrested in metaphase, contained micronuclei of condensed chromosomes, and, when washed with drug-free medium, began dividing within 90 min, producing daughter cells of irregular sizes with abnormal nuclei (22).

The asymptotic shape of survival curves after *in vitro* and *in vivo* exposures to VCR and VLB has been interpreted as meaning that the *Vinca* alkaloids act on proliferating cells primarily in one portion of the cell cycle. The concentration-effect relationships of survival curves obtained for agents that act uniformly throughout the cell cycle are usually first order with respect to drug concentration. Asymptotic survival curves have been obtained for cytotoxicity of the *Vinca* alkaloids against neoplastic (56, 57) and normal (56, 58) proliferating cells and have been attributed to action during mitosis (56, 58). Similar asymptotic survival curves were seen for normal bone marrow cells after treatment of mice with either VCR or VLB (56). Asymptotic survival curves were also observed for mouse lymphoma cells after treatment of mice with VLB or VCR (56).

Despite many reports that *Vinca* alkaloids are synchronizing agents (59, 60), the evidence that inhibition of mitosis is reversible is inconclusive. Metaphase-arrested cells appear unable to resume normal growth after removal of drug. A comprehensive review (16) of clinical and animal studies in which the use of VCR as a synchronizing agent had been proposed found no convincing evidence of synchronization of cells with subsequent normal growth. In most cases, metaphase-arrested cells died; sometimes cells became polyploid, and it was shown (22) that such cells usually undergo abnormal division if they divide at all. A recent study (24) in which the question of reversibility was addressed during VCR treatment of

tumor-bearing mice demonstrated that JB-1 ascites tumor cells arrested in metaphase by VCR subsequently died.

Although mitosis is a major target of cytotoxicity of the *Vinca* alkaloids, non-mitotic cells are also sensitive to these drugs. VCR had a greater killing effect on non-proliferating cultured human lymphoid SK-LN cells (61) and Chinese hamster ovary cells (62) than on proliferating cells. VCR and VLB were cytotoxic during interphase as well as during mitosis against synchronized HeLa and Chinese hamster ovary cells (63); VLB cytotoxicity was seen when synchronized cells were exposed during the G_1 and S phases of the cell cycle, and VCR cytotoxicity was seen when cells were exposed during S phase. Both interphase and mitotic toxicities were observed when cells of the human leukemia lymphoblastoid line, CCRF-CEM, were exposed for 2 hr to 100 nM VCR or VLB; vesicles were released from both interphase and mitotically-arrested cells, and cells eventually lysed (64). Interphase death is also suggested from studies of the effects of VCR treatment on circulating lymphoblastic cells in a patient with acute lymphocytic leukemia (65).

In summary, the *Vinca* alkaloids are antimitotic agents, and cells have been shown to lose proliferative capacity following extended mitotic arrest. Lysis of cells in interphase has also been demonstrated in some cell lines exposed to *Vinca* alkaloids. The phase of the cell cycle in which the *Vinca* alkaloids exert their cytotoxic effects varies, depending on cell type and proliferative state, and the concentrations of drug used. Interphase toxicity occurs at higher drug concentrations than those that result in mitotic arrest.

C. Interaction of Vinca Alkaloids with Microtubules

Inhibition of mitosis by the *Vinca* alkaloids is due to a specific interaction with tubulin. The latter is the structural protein of microtubules and is a heterodimer composed of two unlike polypeptides, α and β (66). The molecular

weights of these subunits range between fifty and sixty thousand daltons, depending on the species of origin and method of determination. Microtubules are spiral, cylindrical structures of variable length, with a depth of 8 nm per complete turn of the helix. The number of tubulin subunits per turn ranges from 10 to 14, depending on the species from which the microtubules were isolated, but is usually 13. Assembly and disassembly of microtubules occurs by orientation-dependent addition of tubulin heterodimers at one end of microtubules and dissociation from the opposite end (67). Net assembly occurs when the rate of tubulin polymerization at one end exceeds the rate of depolymerization at the other end. When tubulin heterodimers to which *Vinca* molecules are bound associate with the assembly end of microtubules, polymerization ceases, and because disassembly continues at the opposite end, microtubules eventually completely depolymerize. Binding of *Vinca* alkaloids to about 1% of the total tubulin content of a cell can half-maximally inhibit microtubule formation (68).

During mitosis, microtubules attach to condensed chromosomes and "pull" them apart, towards opposite ends of the cell (69). Chromosome movement is thought to occur by a sliding process, in which microtubules attached to particular chromatids and to either pole of the mitotic apparatus move along microtubules that extend the length of the cell (69). In a *Vinca*-treated cell, this process does not take place. The binding of *Vinca* alkaloids to tubulin inhibits assembly of microtubules and formation of the mitotic apparatus.

Owellen *et al.* (25) studied the binding of VCR and VLB to tubulin isolated from porcine brain and free of microtubule-associated proteins. Rates of binding of VLB and VCR to purified tubulin were comparable, and Scatchard analyses of drug-binding at equilibrium gave association constants for interaction of VLB and VCR with tubulin, respectively, of 6.0×10^6 M^{-1} and 8.0×10^6 M^{-1}. Studies of VLB binding to rat brain tubulin indicate 2 binding sites per heterodimer. with Ka

values for VLB of 6.2 x 10^6 M^{-1} and 8 x 10^4 M^{-1} (70). Binding of VLB to "high affinity" sites inhibited *in vitro* polymerization of rat brain tubulin, whereas aggregation and precipitation of tubulin resulted from binding of VLB to "low-affinity" sites. VCR and VLB were equally active in blocking *in vitro* polymerization of tubulin purified from beef brain (26), giving rise to the suggestion that differences in clinical activity of VCR and VLB are not due to differences in their action on microtubules.

Some authors have suggested that microtubule-associated proteins may limit disruption of existing microtubules by *Vinca* alkaloids (26, 71). In studies of polymerization of tubulin in the presence of VCR and VLB, microtubule-associated proteins were necessary for spiral structures to "uncurl" from preformed microtubules, and depolymerization of microtubules was not observed. However, these effects were seen at drug concentrations (10 μM) that are not pharmacologically relevant. Binding of *Vinca* alkaloids to purified microtubule-associated proteins was not assayed. The disruptive effect of *Vinca* alkaloids on microtubules is probably due to inhibition of microtubule elongation in these "steady-state structures" rather than to disaggregation of existing microtubules (68).

In summary, VCR and VLB inhibit microtubule-dependent cellular functions, including mitosis, by preventing assembly of microtubules. VCR and VLB have similar, if not identical, association constants for high-affinity binding to purified tubulin (25), suggesting that they are equally active against their primary biochemical target. Microtubule-associated proteins may alter binding to microtubules, but this has not been established.

D. Cellular Uptake of Vinca Alkaloids

Results of limited studies suggest that rates of uptake and release of VLB and VCR differ in several cell types.

When HeLa cells were exposed to cytotoxic concentrations of [^{3}H]VCR and [^{3}H]VLB, the amount of VLB taken up by cells was about 3 times that of VCR, and VLB was released from cells more rapidly than was VCR (72). The concentration of VLB used for drug exposure was 3-fold greater than that of VCR. Uptake and release of [^{3}H]VCR and [^{3}H]VLB were studied in cultured leukemia L1210 cells at concentrations that were cytotoxic during continuous exposure and after short exposures (13). Significantly greater amounts of VLB than of VCR were associated with cells after 4-hr exposures to equal concentrations of either drug, and when cells were transferred to drug-free medium after 4-hr exposures, VLB was released more rapidly than VCR. In cells "loaded" with the same amount of either drug, VLB was lost more rapidly and to a greater extent than VCR over a period of 3 hr. Uptake of both VCR and VLB by L1210 leukemia cells was dependent on the pH of culture fluids, increasing as pH increased.

Rat platelets exposed to equal concentrations of [^{3}H]VCR and [^{3}H]VLB accumulated VLB at higher rates than VCR (27). Although platelets accumulated the same amount of either drug at equilibrium, steady-state levels of VLB were reached within 1 hr, whereas those of VCR were not reached until 10 hr. When platelets were washed and resuspended in drug-free medium, VCR was retained by platelets, and 10-20% of VLB was lost with each successive wash. Similar results were found for rat node lymphoma cells (137, 27), which lost about 50% of cell-associated [^{3}H]VLB, but no [^{3}H]VCR, with successive washes.

Differences in uptake of the *Vinca* alkaloids into desheathed cat sciatic nerve have also been observed (73). In contrast to results obtained in uptake studies with cultured cells and platelets, uptake rates of VCR were greater than those of VLB.

Omission of Ca^{++} from the medium significantly reduced the VCR uptake rate without affecting that of VLB. The uptake of VCR was competitive with respect to VLB, suggesting interaction of the two drugs at the same site, presumably neurotubules. These differences in uptake correlated with biological activity (and *in vivo* neurotoxicity) in that VCR was more effective than VLB in blocking axoplasmic transport in desheathed sciatic nerve.

Thus, with one exception, results of uptake studies with several cell types indicate that uptake and release of VLB is more rapid than that of VCR. When drug incubation periods were sufficiently long that steady-state levels of accumulation were reached, the amounts of cell-associated VLB and VCR were the same. In nervous tissue uptake of VCR was more rapid than that of VLB.

E. Mechanisms of Resistance to Vinca Alkaloids Related to Drug Uptake

There are numerous examples of cultured cell lines and transplantable tumors that were selected for resistance to a particular antiproliferative drug and are cross resistant to a variety of structurally unrelated drugs. Examples of "pleiotropic drug resistance" are presented in Table 2. Pleiotropic drug resistance has most often been found for actinomycin D, adriamycin, daunorubicin, and the *Vinca* alkaloids. Resistance has been correlated with reduced drug uptake (78, 80), possibly resulting from altered membrane permeability (85, 86). Some authors have suggested that cells possess a membrane transporter that mediates entry or exit of such drugs (39, 40), whereas others have concluded that these drugs cross membranes only by passive diffusion (87). The theory that has drawn the most interest is that of an energy-dependent, mediated system of drug efflux across the plasma membrane.

The concept of an energy-dependent efflux system has arisen from observations of the effects of inhibitors of energy metabolism on net uptake of drug in resistant cells. When energy metabolism is normal, net uptake of drug

[1] Abbreviations used are: VCR, vincristine; VLB, vinblastine; VDS, vindesine; ADR, adriamycin; DNR, daunorubicin; VP-16, etoposide; VM-26, teniposide; ACT-D, actinomycin D; CLC, colchicine; MAYT, maytansine; MITHRA, mithramycin; PURO, puromycin; MITO-C, mitomycin C; Dem., demecolcine; CYTO-B, cytocholasin B.

[2]Cell lines were: mouse leukemia P388, human erythroid leukemia K562, human lymphoblastoid leukemia CCRF-CEM, mouse leukemia L5178Y, Ehrlich ascites mouse tumor cells, mouse leukemia L1210, Ridgway osteogenic mouse sarcoma (ROS), mouse tumor MDAY-K2, Chinese hamster cells (CH), and mouse fibroblastoma 3T3FL.

[3]Cross-resistant only at low concentrations.

Table 2

Examples of Pleiotropic Drug Resistance[1]

Resistant Sub-line[2]	Selected for Resistance to:	Cross-resistant to:	Ref.
P388	VCR	VCR, VLB, ADR,	74,75
		DNR, VP-16	76,77
	ADR	VCR, VLB, VDS, ACT-D	78
		MAYT, MITHRA, VM-26, VP-16	
K562	VCR	VLB, VDS	79
CCRF-CEM	VCR	VLB, VDS, MAYT, CLC,	35
		VM-26, VP-16	
L5178Y	VCR	VLB[3], VDS[3]	80
		ADR	
Ehrlich ascites	VLB, VCR	VDS, ADR, DNR	81
	DNR	VCR	39,41
L1210	VDS	VCR[3], VLB[3]	80
		ADR, ACT-D	
	VM-26	VCR, ADR, ACT-D, VP-16	82
ROS	ADR	VCR	83
MDAY-K2	ADR	DNR	84
CH	ADR	VCR, VLB, DNR, MITHRA,	85
		PURO, MITO-C, Dem.	
3T3FL	MAYT	VCR, ADR, CLC, CYTO-B	40

occurs at reduced rates and to a lesser extent in drug-resistant, than in drug-sensitive, cells. However when energy metabolism is inhibited, the differences in uptake between drug-sensitive and resistant cells are eliminated. Efflux of [^{3}H]VCR from VCR-resistant (AH66) rat hepatoma cells was more sensitive to 2,4-dinitrophenol in glucose-free medium than that of VCR-sensitive (AH13) cells, suggesting the existence of an energy-dependent efflux system that was greatly amplified in resistant cells (42). A similar phenomenon was found in studies with VCR-sensitive and resistant P388 mouse leukemia cells (43). The differences in net uptake of [^{3}H]actinomycin D and [^{3}H]VCR in adriamycin-sensitive and resistant Ridgway osteogenic sarcoma cells (83) and of [^{3}H]VCR and daunorubicin in daunorubicin-sensitive and resistant Ehrlich ascites cells (39, 41) have also been attributed to energy-dependent efflux.

An alternative explanation for energy-dependent differences in net uptake of drug seen between drug-resistant and drug-sensitive cells has been proposed (35). After energy depletion, net uptake of [^{3}H]VLB by human lymphoblastoid leukemia CCRF-CEM cells was reduced in VLB-sensitive and increased in VLB-resistant cells; restoration of energy metabolism had opposite effects on net uptake of VLB in drug-sensitive and resistant cells. The interpretation of these results was that drug-resistant cells possess an energy-dependent system, perhaps involving phosphorylation, that reduces the number of binding sites available for interaction with drug. Energy depletion of resistant cells makes these sites available for drug binding, and restoration of energy metabolism "blocks" drug binding sites, thereby eliminating trapping of drug in cells.

It has recently been shown (75, 88, 89, 90, 91, 92) that pleiotropic drug resistance can be circumvented with calcium channel blockers such as verapamil. Also, sensitivity of cells to drug has been reduced with the use of some compounds, including ruthenium red (93, 94). These observations have been

interpreted in a number of ways, but they may all be linked to an energy-dependent system of resistance. It is possible that blockage of Ca^{++} efflux by ruthenium red makes cells resistant to VCR by increasing the intracellular Ca^{++} concentration and enhancing Ca^{++}-dependent drug efflux of VCR (93, 94). Verapamil, and other agents that block Ca^{++} uptake and diminish intracellular Ca^{++} content, render cells more sensitive to drugs, including the *Vinca* alkaloids (75, 88, 89, 90, 91, 92). The association of high intracellular Ca^{++} with resistance (90, 95) suggests that Ca^{++} metabolism may be involved in pleiotropic drug resistance. It is also possible that the calcium channel blockers eliminate resistance (a) by increasing membrane fluidity, allowing greater uptake of drug (96), or (b) by directly inhibiting efflux of drugs, either competitively or by binding to a Ca^{++} site on the putative efflux protein (97, 98).

Increased expression of large molecular weight membrane glycoproteins accompanies acquisition of pleiotropic drug resistance in several cell types (82, 99, 100, 101, 102, 103, 104). Of particular interest has been the identification of a 150-190 kd glycoprotein that is present in much greater amounts in the membranes of VCR-resistant, than of sensitive, CCRF-CEM lymphoblastoid and Chinese hamster cells (99, 100, 102, 103). It is possible that the 170-190 kd membrane glycoprotein is the product of an amplified gene (101, 102, 103) that is responsible for the greater efflux of drugs seen in resistant cells. Neither of these possibilities has been proven.

F. Pharmacokinetics

1. Methods

Pharmacokinetic studies in animals and humans have been conducted with the *Vinca* alkaloids using a variety of techniques. The earliest studies with VLB

employed radiolabelled drug prepared by an exchange reaction with concentrated tritium gas (31). In 1968, Greenius *et al.* (105) prepared [^{3}H]-4-acetyl-vinblastine by a method that is still widely used today: chemical deacetylation of VLB followed by reacetylation by reaction with [^{3}H]-acetic anhydride. The first pharmacokinetic study with VCR made use of a bioassay in which serum from patients given VCR was assayed for antiproliferative activity against cultured cells (15). Radiolabelled VCR for use in pharmacokinetic studies was first produced in 1972 by exchange with [^{3}H]-trifluoroacetic acid using a platinum catalyst (33). This method can also be used for preparation of [^{3}H]VLB (34).

A drawback of the use of radiolabelled alkaloids is the necessity of separating possible metabolites from parent drug to effectively quantitate drug levels in serum or tissues. Some metabolites, although biologically inactive, may contribute significantly to the radioactive content of samples. VCR and VLB decomposition products and/or metabolites have been separated by thin layer and high performance liquid chromatography. The latter method is preferred for a number of reasons, including the reduced likelihood of spontaneous degradation, particularly of VCR, during the separation procedures (30).

Recently, immunological methods have been described for assay of *Vinca* alkaloids in body fluids (29, 106). Monoclonal antibodies have been prepared that recognize only parent drug (29, 106). Such assays are highly sensitive and can detect drug levels as low as 0.5 nM (11, 106, 107).

2. Animal Studies

VCR and VLB distribute in a similar manner in body tissues of animals. After equilibration of injected drug with tissues, the greatest accumulations are found in lymphoid tissues, including the spleen and thyroid, and in adrenal glands (28, 29, 30, 31, 32, 33, 108). Moderate levels of drug also distribute into lung, kidney, bone

marrow, and liver. Bile appears to be the major route of clearance of *Vinca* alkaloids (29, 31, 32). VCR is excreted unchanged, whereas VLB appears to be extensively metabolized by liver before excretion in bile (30, 31, 32, 33).

Within 1 to 2 hr of administration of [^{3}H]VLB to rats, platelets contained 60% of total blood radioactivity (34). In another study with rats (105), [^{3}H]VLB was concentrated 5-fold in white blood cells and platelets 2.5 hr after administration of drug. White blood cells of dogs have also been found to concentrate [^{3}H]VLB (108). Platelets contain large numbers of microtubules, and the accumulation of VLB in platelets (109), and probably also in lymphoid tissues and leukocytes, is due to binding to tubulin. The greatest concentration of radioactivity 24 hr after injection of [^{3}H]VLB into rats was found in the intestinal contents and urine, followed by liver, blood, and intestinal tissues (29, 32). Radioactivity was recovered from liver and spleen as apparent metabolites and from lymphoid tissues as unchanged drug (28). Twenty percent of total injected radioactivity was found in bile, mostly as metabolites of VLB (32). Following administration of [^{3}H]VLB to dogs, half of the radioactivity present in urine and nearly all the radioactivity in feces was in the form of metabolites or products of decomposition (108).

[^{3}H]VCR was found to distribute in the bodies of rats in much the same way as VLB (30, 33). In contrast with the extensive metabolism of VLB by liver and bile (31), up to 90% of radioactivity recovered from bile and urine was unchanged VCR (30, 33). In dogs, 4 hr after administration of [^{3}H]VCR, most of the label was localized in the spleen and lungs, and bile was the major route of excretion (30). Metabolism was not reported.

In summary, bile appears to be the major route of clearance of *Vinca* alkaloids from animals. VCR is excreted mostly unaltered, and VLB is extensively metabolized (liver and possibly spleen in rats) before being excreted. Although both drugs localize in lymphoid tissue, their relative localization in nervous tissues and

bone marrow, the dose-limiting tissues for toxicity, have not been determined.

3. Clinical Studies

Data from pharmacological studies of the *Vinca* alkaloids in humans are summarized in Table 3. As in animals, bile is the major route of clearance from humans. VLB appears to be extensively metabolized by liver before being excreted, and 50-90% of VCR is excreted unchanged (17, 30, 31, 32, 33, 108, 110). After administration of [^{3}H]VLB, greater than 99% of radioactivity in feces and between 10 and 33% in urine was present as metabolites or decomposition products (37). Metabolism of VLB occurs primarily in liver, and desacetylvinblastine, the major metabolite recovered in urine and feces, is more cytotoxic against cultured cells than VLB (17).

Clearance of the *Vinca* alkaloids from serum has a triphasic pattern (8, 11, 17, 110, 111), and the first two phases (α and β), which include biliary clearance and tissue uptake, have the same half-lives for VCR and VLB. However, the third phase (γ) appears to be considerably longer for VCR than for VLB, with half-lives, respectively, of 85 $\pm$ 70 hr and 25 $\pm$ 7 hr (11).

Following 1-hr infusions in leukemic children, plasma VCR levels were greater than 50 nM for up to 2 hr in most patients and remained between 5 and 50 nM for at least 4 hr (15). In adult patients given VCR and VLB (the latter at doses 5-fold greater than those of VCR), the concentrations of VCR and VLB, respectively, dropped from 70 to 7 nM and 150 to 10 nM between a few min and 4 hr after injection (11). In a similar study (17), the plasma concentration of VLB declined sharply within a few min of injection, from 500 to 100 nM, with a further decay to about 2 nM by 4 hr. For both VCR and VLB, serum levels of about 100 nM represent less than 5% of the original dose (16). Within minutes of injection, most of the drug is rapidly distributed into interstitial fluids and fatty

Table 3
Vinca Alkaloid Clearance from Humans

Drug	Ref.	Method	Time (hr)	Excretion (% of Total Dose)			Plasma Clearance ($t_{0.5}$, min)			No. of patients
				Feces	Urine	Bile	α	β	γ	
VCR	11	RIA[1]					4.62±2.04	136±90	5100±4130	4
	8	RIA	90		37		2.6	41	1531	4
	110	[^{3}H][2]	3		6.5					4
			24	33						
			72	69	12					
	111	RIA					1.9	19.2	1359±1002	14
	10[4]	[^{3}H]	24			21.7				1
			72	2.6	26	28				
	112	RIA					2.9±0.6	23.7±8.5	1080±350	4
	107	RIA							864-2250	6
	12	RIA	4		6.1±3.0		3.37±0.72	155±18		3
			12		8.6±5.2					
			24		9.6±5.1					
VLB	11	RIA					3.72±2.40	98.4±20	1488±450	3
	37	[^{3}H]	72	33±11	21±3		4.52±0.37	190±7		2
	17	RIA					3.9±1.46	53±13	1173±65	3

[1]RIA, radioimmunoassay
[2][^{3}H], radiolabelled drug
[3]patient had cannulated bile duct

tissues, followed by a slow equilibration into the "deep tissue compartment", which is thought to represent binding to intracellular protein, presumably tubulin.

Analysis of the pharmacokinetic data of Table 1 (not shown) has established that the "apparent volume" of the tissue compartments into which VLB distributes after injection is 3 to 4 times greater than that of VCR (12, 17). Following i.v. bolus injections, VLB apparently equilibrates rapidly in locations that are not accessible to VCR. Since VLB is more lipophilic than VCR, there may be greater localization of VLB, than VCR, in fatty tissues and in membranes.

It is apparent from the few long-term studies of clearance that, after 72 hr, 20-50% of the total administered drug is retained within the body (10, 37, 110). VCR and VLB are "sequestered" in tissues and are gradually released into blood over a period of several days, during which time serum levels drop from 10 to 1 nM within 24 hr and then slowly decline below levels of detection after about 4 days (11, 17, 107, 110, 111, 112). The more rapid decline of serum levels of VLB than VCR during the third (γ) clearance phase undoubtedly arises from its different distribution in the body and contributes to the differences in therapeutic activity of the two drugs.

III. Human Promyelocytic Leukemia HL-60

The human leukemia cell line HL-60 was derived from peripheral blood leukocytes of an adult female with acute promyelocytic leukemia (14). After 8 days in primary culture, virtually all of the surviving cells were positive for peroxidase and Sudan black staining, indicating the presence of myelocytic cells. After subculture, proliferating HL-60 cells had population doubling times of 55-60 hr, and 17% of cells exhibited a "differentiated" phenotype. The cell line had a modal chromosome number of 44 and was negative in tests for Epstein-Barr virus nuclear antigen. It was later discovered that HL-60 cells could be chemically induced to differentiate to either monocyte and macrophage-like cells or to granulocytic cells (18, 19). A selected list of inducers of differentiation of HL-60 cells is presented in Table 4.

Treatment of HL-60 cells with tetradecanoylphorbolacetate (TPA) stimulates expression of a number of "markers" of normal monocytes, including non-specific esterase (114, 120, 121), acid phosphatase (120, 121, 122), ingestion of latex beads (120), adherence to plastic (18, 121), NADase (121), 5'-nucleotidase (114), and complement C3 and Fc receptors (123). TPA also causes repression of two granulocytic markers, specific esterase (120) and myeloperoxidase (114, 121). Monocyte characteristics that TPA does not enhance in HL-60 cells are superoxide generation, ingestion of *E. coli*, hexose monophosphate shunt, complement C2 secretion, and reduction of nitroblue tetrazolium (114).

The best documented inducer of differentiation of HL-60 cells in the direction of granulocytic cells is dimethylsulfoxide (DMSO). Retinoic acid has also been shown to be a potent inducer (115). The granulocytic markers that are expressed in DMSO-treated HL-60 cells are lysozyme release (121), superoxide generation (113, 117), phagocytosis (19, 117), chemotaxis (113), β-glucuronidase activity (117), hexose monophosphate shunt activity (117), and complement C3 and Fc receptors (113, 123). In most instances, the activities of these markers are not

[1]Abbreviations: DMSO, dimethylsulfoxide; TPA, 12-O-tetradecanoyl-phorbol-13-acetate; DMF, dimethylformamide; HMBA, hydroxymethylbutyric acid; ACT-D, actinomycin D; MTX, methotrexate.

[2]Morphological assessment

[3]NBT, nitroblue tetrazolium. Reduction of NBT is due to the presence of O_2^-, a product of oxidase activity in mature white blood cells (granulocytes and macrophages) (119). Normal granulocytes and monocytes from peripheral blood reduced NBT, respectively, by >90% (113) and 97±3% (114).

Table 4
Induction of HL-60 Differentiation

Ref.	Inducer[1]	Length of treatment (days)	% mature cells[2]	% NBT—reducing cells[3]
113	0		8-12	8
	1.12 % DMSO	6	75-85	72
114	0			5±2
	16 nM TPA	4		5±3
19	0		11	
	1.15 % DMSO	6	73	
	1.30 % DMSO	6	94	
	60 mM DMF	6	84	
	0.6 mM Butyric acid	6	58	
115	1.14 % DMSO	6	96	
	0.5 mM Butyric acid	6	86	
	1 μM Retinoic acid	6	94	92
	2.0 mM HMBA	6	91	
116	0		7	
	2 mM HMBA	6	95	97
	5 mM Hypoxanthine	6	86	90
	1.3 % DMSO	6	75	80
	5 ng/ml ACT-D	6	85	
117	0		4	10
	1.3 % DMSO	6	59	97
	1.3 % DMSO	9	94	97
118	0		6	6
	25 μM 3-deazauridine	6	96	94
	1 μM pyrazofurin	6	63	57
	14 nM MTX	6	35	31
	1.2 % DMSO	6	89	89

equal to those of normal granulocytes. Collins *et al.* (19) noted the following morphological changes in HL-60 cells following induction by DMSO: "smaller size, decreased nuclear/cytoplasmic ratio, less prominent cytoplasmic granules, marked reduction or complete disappearance of nucleoli, pyknotic changes in nuclear chromatin, and marked indentation, convolution, and segmentation of the nuclei".

Reports of the effects of *Vinca* alkaloids on HL-60 cells are limited. After 6-day exposures of HL-60 cells to VCR, there was a 2-fold increase in the percentage of myelocytic cells (from 8% to 18%) as proliferation rates were reduced by about half (116). It is likely that nonproliferating, monocytic cells were present in cultures when drug exposures were initiated and survived prolonged exposures to VCR.

The transformed state of HL-60 cells appears to be related to the expression of a number of oncogenes. The *ras* oncogene codes for a 21 kilodalton protein that has been found in HL-60 cells (124, 125). The *myc* oncogene is present in HL-60 cells in about 20-30 copies per cell, 3-fold greater than the number in normal lymphocytes (124, 125, 126). When differentiation of HL-60 cells is induced by DMSO, retinoic acid, or TPA, expression of the *myc* oncogene declines. Expression of the *myb* oncogene (also referred to as *amv*) also declines in DMSO or retinoic acid-induced HL-60 cells (127), whereas expression of the *abl* oncogene does not change (125). The relationships between expression of the *myc*, *ras*, and *myb* oncogenes and proliferation of HL-60 cells are not known.

IV. Materials and Methods

A. Cell Culture

Human promyelocytic leukemia HL-60 cells (14) were a generous gift from Dr. R. Gallo, NIH, Bethesda, MD. HL-60 cells were routinely maintained as suspension cultures in the absence of antibiotics in Roswell Park Memorial Institute medium 1640 (RPMI 1640) supplemented with 15% fetal bovine serum (FBS). Stock cultures were kept at 37° in a humidified atmosphere of 5% CO_2 in air, and were subcultured 3 times per week by diluting to 10^5 cells/ml, or 0.5 x 10^5 cells/ml over weekends. Preliminary experiments reported in reference 13 were conducted with the original cell line, which had a doubling time of 36-48 hr and a cloning efficiency in soft agar culture of 30%. The experiments presented here were conducted with a clonal derivative (HL-60/C1), which was begun from a single colony. HL-60/C1 cells were found to grow exponentially between subculture periods with a doubling time of 24 hr and exhibited a cloning efficiency of 50%. Ampules of HL-60/C1 cells were stored in liquid nitrogen, and after 30 to 40 subculture generations, new cultures were restarted from frozen stocks, demonstrated to be free of *Mycoplasma* (Dr. J. Robertson, Medical Bacteriology, University of Alberta). For experimental purposes, cells were subcultured daily until use by diluting to 10^5 cells/ml, and, unless otherwise noted, culture fluids contained 4 mM Hepes buffer (pH 7.4), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cell numbers were determined by enumerating with an electronic particle counter (Model ZB, Coulter Electronics, Hialeah, FL).

To determine the effects of drugs on cell proliferation rates, suspensions of exponentially proliferating cells (2 x 10^5 cells/ml) were combined with equal volumes of fresh growth medium containing VCR or VLB at twice the concentration to be tested. For continuous exposures, cell concentrations were determined in duplicate

cultures at 0, 24, and 48 hr, intervals during which untreated cells grew exponentially. For 4-hr exposures, the exposure to drug was stopped by centrifuging cells (120 g, 8 min), washing once in fresh, drug-free growth medium, and resuspending at approximately 10^5 cells/ml in fresh medium. Cell concentrations were determined at 0, 24, and 48 hr after resuspension of cells in drug-free medium. Cell recoveries following centrifugation, washing, and resuspension were about 85-90% for all cultures.

Drug effects on reproductive viability were determined by assay of colony-forming ability in soft agar using a modification of an established procedure (128). HL-60/C1 cells were exposed for 4 hr, as described above for proliferation experiments, and, after being washed with drug-free growth medium, were resuspended and diluted in cloning medium. Cloning medium consisted of RPMI 1640 supplemented with 15% FBS, 10% "conditioned medium" and gentamicin (50 μg/ml). Conditioned medium was obtained by filtration (0.22 μm Millipore filter, Millipore Corporation, Bedford, Mass.) of spent medium from cultures of HL-60/C1 cells (initial concentration 10^5 cells/ml) that had been proliferating exponentially for 48 hr and was either used immediately or stored at 4° overnight. Soft-agar cultures were established in 5 ml cloning medium (100-200 cells/6-cm petri plate), to which had been added 0.13% agar Noble. Such cultures were incubated 14 days at 37° in a humidified atmosphere of 5% CO_2 in air. Colonies were approximately 1 mm in diameter, and were counted using a low magnification (7x) microscope.

Drug effects on viability were also determined by dye exclusion. Suspensions (0.5 ml) of HL-60/C1 cells in growth medium (1-2 x 10^5 cells) were mixed with 0.25 ml of trypan blue solution (0.5% w/v in physiological saline) and incubated 15 min at 37°. Cells were scored for staining by observing wet mounts using an inverted microscope.

B. Cell volume determinations

Volume determinations of HL-60/C1 cells were obtained using a Model Zf Coulter counter fitted with a 100 μm aperture tube in conjuction with a 100-channel particle size analyzer (Channelyzer II, Coulter Electronics Inc.) interfaced with an Apple II+ computer (Apple Computer Inc., Cupertino, CA). The channelyzer was calibrated with polystyrene microspheres of 10.08 μm diameter (Coulter Electronics Inc., Oakville, Ont.), and volume distributions were analyzed using software obtained from Coulter Electronics Inc. A shape factor of 1.5, corresponding to a spherical non-conducting particle, was used for all determinations, and mean and modal cell volumes were calculated. Table 5 demonstrates that the volume of HL-60/C1 cells was stable for up to 90 min in physiological saline (0.15 M NaCl). All determinations of cell concentration and cell volume were routinely concluded within 20 min of introducing the cells to saline. The cell volume was used in some experiments to estimate "apparent" intracellular concentration[1] of drug for calculation of ratios of intra and extracellular concentrations. Since cellular drug content was an average for cells in a particular culture, the mean cell volume was used to calculate such ratios.

C. DNA distributions

The relative DNA content of HL-60/C1 cells was determined by flow cytometry using mithramycin, a fluorescent DNA-binding agent, and a modification of previously described procedures (129, 130, 131). Portions of cell suspensions of sufficient volume to give 1.5-2 x 10^6 cells were centrifuged (120 g, 8 min), and the pellets were resuspended in 1 ml of ice-cold saline. Cells were then fixed by adding 5 mls of cold 70% ethanol, dropwise at first to avoid clumping. Fixed cells

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[1]Since some of cell-associated drug was bound to intracellular components (e.g., tubulin), the values obtained for intracellular drug concentrations are overestimates.

Table 5

Stability of HL-60/C1 Cells in Physiological Saline:

Cell Volume Determinations

Time (min)	Mean Volume (fl ± S.D.)	Modal Volume (fl)
2	1161±354	1000
15	1090±329	873
30	1084±332	937
45	1062±325	937
60	1067±325	937
90	1068±334	905

Exponentially proliferating HL-60/C1 cells were established in culture (10^5 cells/ml) and incubated at 37° for 24 hr. One-ml portions of cell suspensions were added to 19 ml of physiological saline, and mean and modal cell volumes were determined at the times indicated as described in the text. The standard deviations presented are those of individual distributions. One determination was performed for each time point.

could be kept in ethanol in a refrigerator (4°) for several weeks before further processing. Immediately before beginning the staining procedure, the ethanol-cell suspensions were centrifuged and the pellets were washed once in 5 ml of saline to remove excess ethanol, which was found to alter fluorescence intensity, and resuspended in 0.75 ml of mithramycin (100 μg/ml) staining solution. The latter was prepared by dissolving 2.5 mg of mithramycin in 5 ml of distilled H_2O, then diluting 1:4 with 0.1 M Tris-HCl (pH 7.6), 0.1 M NaCl, and 15 mM $MgCl_2$. Cells were allowed to stain in the dark for 30 min, after which the fluorescence intensity was stable for several hr. Fluorescence intensity, a measure of relative DNA content of mithramycin-stained cells, was determined with a Coulter Electronics Epics V fluorescence activated cell sorter (Coulter Electronics Inc., Oakville, Ont.) equipped with an argon laser (peak emission, 457 nm) used at an output of 200 mW. Mithramycin has peak excitation and emission wavelengths, respectively, of 421 nm and 575 nm. Red flourescence was detected after sequential filtering with a 515-nm absorbance filter and a 515-nm interference filter, a 560-nm dichroic filter, and a 570-nm long-pass filter. Data were collected on a Multiparameter Data Acquisition and Display System (MDADS). Charts of DNA distributions were produced using the GRAFP2 program (Coulter Electronics EASY I software) on a Terak 8510 computer interfaced with a Terak 8600 Graphics display terminal (Scottsdale, AZ).

D. Purification of [^{3}H]VCR and [^{3}H]VLB

[^{3}H]VCR and [^{3}H]VLB were produced by a catalysed reaction in which tritium atoms of [^{3}H]trifluoroacetic acid exchanged with hydrogen atoms of the 17-position of the indole heterocycle[1] (33, 34). Radiochemicals were supplied in methanol and were stored at -20°. [^{3}H]VCR and [^{3}H]VLB were purified within 4

[1]Dr. J. Moravek, personal communication

days of experiments by high performance liquid chromatography (HPLC) using a modification (Table 6) of a solvent system previously described (132). The column used for separation was a reverse phase partisil 10/25, ODS-3 (Whatman Chemical Separations, Clifton, NJ), which was attached to an SP8000A pump and gradient generator (Spectra-Physics, Santa Clara, CA). The detection system was either a Spectra-Physics SP8310 fixed wavelength detector (254 nm) or SP8400 variable detector (259 nm). Portions of [^{3}H]VCR and [^{3}H]VLB in methanol (or previously purified drug in H_2O) were dried using a rotary evaporator. The residue was resuspended in 230 μl of H_2O, 200 μl of which were injected onto the column using a WISP 710B automatic injector (Waters Associates, Milford, Mass.). For determination of the radioactive content of eluates, 1-ml fractions were collected directly into scintillation vials using an LKB automatic fraction collector (LKB-Produkter AB, Bromma, Sweden). To each vial were added 8 ml of a xylene:detergent scintillant (133), and the resulting mixtures were left in the dark for 24 hr before determination of radioactive content by liquid scintillation counting (Model LS 7500, Beckman Instruments, Irvine, CA).

The elution profiles of Figure 2 are representative of those usually obtained for [^{3}H]VCR and [^{3}H]VLB during purification. The parent compounds were clearly separated from their products of radioactive decay. VLB, which is more hydrophobic than VCR, exhibited a longer retention time.[1] None of the degradation products present in stock solutions of radiochemicals was identified. Figure 3 demonstrates the success of the purification procedure in producing homogeneous preparations of [^{3}H]VCR and [^{3}H]VLB. Three days after purification, analysis of these preparations indicated that essentially all of the radioactivity co-eluted with unlabelled standards.

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[1]The octanol:water partition coefficients at pH 7.4 of VLB and VCR are 2000 and 160, respectively (38).

Table 6

Gradient System[1] for HPLC Elution of

[^{3}H]VCR and [^{3}H]VLB

Time (min)	KH_2PO_4 [2] %	Methanol %
0	55	45
12	37	63
30	25	75
36	21	79
40	0	100
50	55	45

[1]Although the gradient system was generally maintained to elute [^{3}H]VCR and [^{3}H]VLB (1 ml/min, 40°) between 17 and 24 min after injection, it was sometimes altered slightly because of changes in column activity and because the mixing system did not siphon the two solvents with equal efficiency.

[2]10 mM, pH 4.9

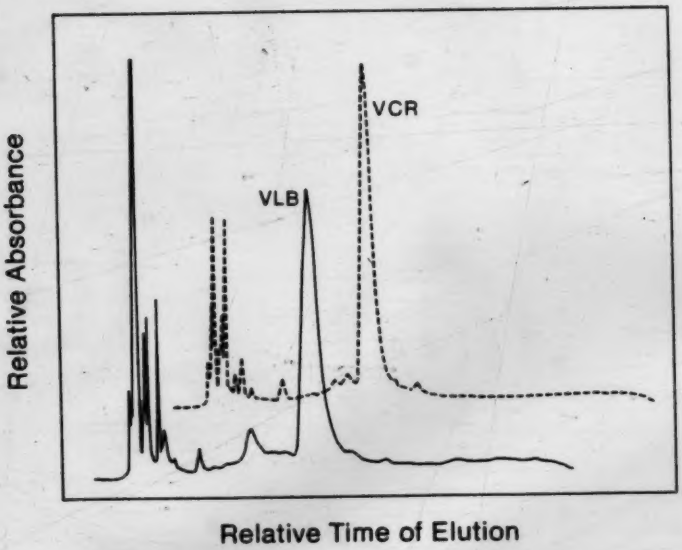


Figure 2. Separation of [^{3}H]VCR and [^{3}H]VLB by HPLC. The tracings represent absorbance at 259 nm and demonstrate the separation of [^{3}H]VCR and [^{3}H]VLB from products of radioactive decay. The separation time was 45 min, and [^{3}H]VCR and [^{3}H]VLB eluted at 18 and 22 min, respectively.

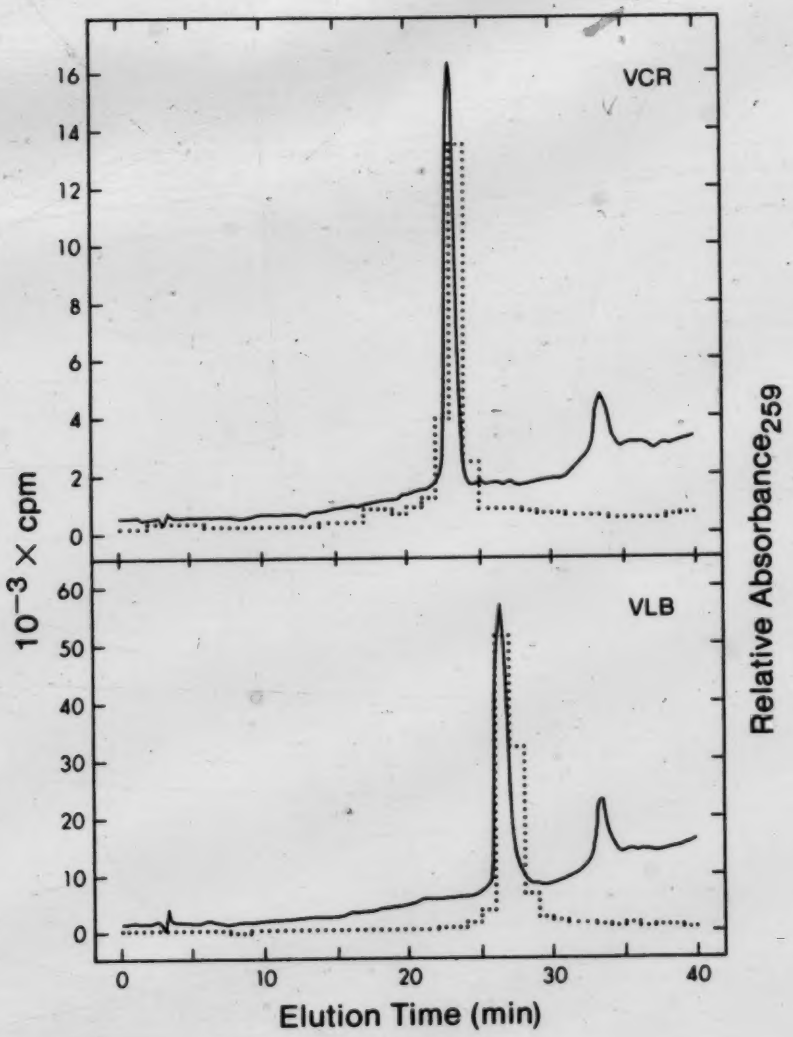


Figure 3. Analysis of [^{3}H]VCR and [^{3}H]VLB purified by HPLC. Solutions containing [^{3}H]VCR or [^{3}H]VLB, together with unlabelled VCR or VLB as carrier, were eluted as described in the text for the purification procedure. Absorbance was monitored at 259 nm. *Dotted lines*, radioactivity (10^{-3} x cpm); *solid lines*, relative absorbance (259 nm).

[^{3}H]VCR and [^{3}H]VLB were supplied with a specific activity of 9-12 Ci/mmol. After purification, the specific activity ranged from 3-7 cpm/μmol, and was usually about 4-5 cpm/μmol, which, with a counting efficiency of 20%, corresponded to 9-11 Ci/mmol. When experiments required concentrations greater than 50 nM, [^{3}H]VCR and [^{3}H]VLB were mixed with nonisotopic VCR or VLB, respectively.

E. Binding of VCR and VLB to serum proteins

The binding of VCR and VLB to proteins present in fetal bovine serum was studied by equilibrium dialysis. The apparatus (Hoefer Scientific Instruments, San Fransisco, CA) had two plastic discs between which the dialysis membrane (molecular weight cutoff, 14,000) was placed. There were eight 200-μl ports around the perimeter of each disc. The two discs were aligned such that each port was opposite a port on the other disc, and the dialysis membrane separated the aligned ports from each other. Dialysis membranes, activated by boiling for 2 min in a solution of 5% Na_2CO_3, were rinsed thoroughly with distilled H_2O. A 200-μl sample of the test solution was added to one member of a pair of ports and 200 μl of RPMI 1640 containing 50 μg/ml gentamicin and 5 mM Hepes (at the appropriate pH) were added to the port on the opposite side. To determine the concentration of free drug on either side of the membrane at equilibrium, some pairs of ports had 15% FBS on both sides of the membrane. The ports were sealed and incubated on a stirring apparatus for 24 hr at 37°. Samples of 100 μl were removed from each port (i.e., both sides of the membrane) and were counted in 8 ml of a xylene:detergent scintillant (133).

Drug binding was assessed in RPMI 1640 supplemented with FBS (2, 5, 10, or 15%), 5 mM Hepes buffer (pH: 5.5, 6, 6.5, 7, 7.5, or 8), and gentamicin (50 μg/ml). [^{3}H]VCR was present at concentrations of 50, 100, 200, or 400 nM,

and [^{3}H]VLB was present at concentrations of 50, 200, 800, 1000, or 3000 nM. The pH of test solutions was adjusted using 6 M HCl or 10 M NaoH.

F. Metabolite studies

Possible metabolism of the *Vinca* alkaloids by HL-60/C1 cells was examined as follows. Cultures established with exponentially proliferating cells (1.5 x 10^5 cells/ml) were incubated for 24 hr in the absence (untreated) or presence of 20 nM [^{3}H]VCR or [^{3}H]VLB, after which cells were collected by centrifugation (120 g, 8 min). One ml of each supernatant was saved and processed for analysis of metabolites as described below, and the remainder was discarded. The cell pellets were washed once with 10 ml of ice-cold saline and were then subjected to 3 successive extractions in 4 ml of ice-cold 95% ethanol. Just before the first extraction, 5 nmol of the appropriate unlabelled drug were added, as carrier, to cell pellets obtained from untreated and drug-exposed cultures. As well, to assess spontaneous decomposition during the extraction procedure, 40 pmol of [^{3}H]VCR or [^{3}H]VLB were added to cell pellets obtained from untreated cultures. After the first extraction, samples were centrifuged at 400 g (3 min, 4°), and after the second and third extractions, samples were centrifuged at 700 g (3 min, 4°). The three supernatants from each sample were pooled in 25-ml round-bottom roto-evaporator flasks, and the ethanol extracts were dried by rotary evaporation. The resulting residues were dissolved in 250 µl H_2O, 200 µl of which were immediately injected onto a reverse-phase partisil column and eluted as described in Section D. One-ml fractions were collected and mixed with 8 ml of xylene:detergent scintillant (133), and, after 24 hr in the dark, the radioactive content of the resulting mixtures was determined by liquid scintillation counting.

Analysis of possible metabolites in culture fluids after 24-hr drug exposures was as follows. Portions (200 µl) of the previously reserved supernatants, obtained

as described above, were incubated with 1 ml of 95% ethanol in microfuge tubes for 1 hr at 4°. The tubes were then centrifuged (12,800 g, 10 min, 4°). The resulting supernatants were dried by rotary evaporation, and the residues were dissolved in 230 μl H_2O for analysis by HPLC as described above for cell extracts. One-ml fractions of eluates were collected for determination of radioactive content as described above.

G. Uptake and release of [^{3}H]VCR and [^{3}H]VLB

Measurements of cellular uptake of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells were conducted under conditions similar to those of proliferation experiments. Cultures were established as described in Section A and, for 4-hr exposures, were incubated at 37° with the caps tightened or, for longer exposures, at 37° in a humidified atmosphere of 5% CO_2 in air with the caps loose. Cell-associated radioactivity for each condition was determined as follows. Triplicate 1-ml samples were removed from cell cultures at graded time intervals and were transferred to 1.5-ml polypropylene microcentrifuge tubes and centrifuged (12,800 g, 1 min). The supernatants were aspirated and discarded, and the cells were washed once by resuspending in 1 ml of ice-cold physiological saline and centrifuging (12,800 g, 1 min). One of the three replicate pellets was resuspended in 1 ml of saline, and the cell concentration was determined with an electronic particle counter. The ^{3}H content of the other two pellets was determined by solubilizing overnight in 100 μl of 1% Triton X-100 (v/v). The microfuge tubes and 8 ml of a xylene:detergent scintillant (133) were placed in scintillation vials, and, after being left in the dark for 24 hr, the radioactive content was determined by liquid scintillation counting.

The effects of pH and serum concentration on uptake of [^{3}H]VCR and [^{3}H]VLB was assessed by modifying the procedure above as follows. Ten ml of exponentially proliferating cultures (3 x 10^5 cells/ml) were centrifuged (120 g, 8

min), and the resulting pellets were resuspended in 8 ml of (a) RPMI 1640 supplemented with 15% FBS and 4 mM Hepes that had been adjusted with 6 M HCl or 10 M NaOH to a pH of 5.5, 6.0, 6.5, 7.0, 7.5, or 8.0 or (b) RPMI 1640 supplemented with 4 mM Hepes (pH 7.4) and 2, 5, 10, or 15% FBS. Uptake reactions were initiated by combining 3-ml portions of cell suspensions in the appropriate medium with 3-ml portions of the same medium containing 100 nM [^{3}H]VCR or [^{3}H]VLB. The resulting cultures were incubated at 37° in sealed tubes that were positioned on a vertically rotating test-tube rack to keep the cells suspended. At 4 hr, five 1-ml samples were removed, and 3 were processed for determination of cell-associated radioactivity by scintillation counting and 2 for determination of cell number, as described above.

To measure rates of drug release, cells were "loaded" with [^{3}H]VCR or [^{3}H]VLB under the conditions described for uptake experiments. After 4 or 24 hr, triplicate 1-ml samples were removed and processed for determination of cell-associated radioactivity, as described above, to obtain "time-zero" values for time courses of drug release. The remainder of each culture was centrifuged (120 g, 8 min), and the supernatants were aspirated and discarded. Drug release was initiated by resuspending each pellet in a volume of drug-free growth medium such that the cell concentration was the same as during the loading procedure. The resulting cell suspensions were incubated at 37°, and at graded time intervals thereafter, triplicate 1-ml samples were removed and cell-associated radioactivity was determined as described above.

H. Uptake and release of VLB by ATP-depleted cells

To determine conditions that would deplete cellular ATP, HL-60/C1 cells were exposed for varying lengths of time to 10 mM sodium azide in glucose-free or glucose-containing (2 μg/ml) RPMI 1640 supplemented with 15% dialyzed FBS.

Serum was dialyzed at 4° by washing once for 8 hr and twice for 24 hr in 20 volumes of saline (Spectrapor dialysis membrane, molecular weight cutoff 3500, Spectrum Medical Industries, Los Angeles, CA) and was sterilized before use by filtration (0.22 μm Millipore filter).

Cellular ATP content was determined by an established procedure (134). Fifty-ml cultures (3 x 10^5 cells/ml) were cooled 3 min on ice and then centrifuged (1000 g, 2.5 min, 4°). The medium was aspirated, and, without resuspension, the pellets were centrifuged again (1000 g, 2.5 min, 4°). After carefully removing as much as possible of supernatants, the pellets (about 1.5 x 10^7 cells) were suspended in 100 μl of 0.4 M perchloric acid. The resulting mixtures were vortexed, incubated on ice for 20 min, with a second vortexing at 10 min, and centrifuged (1000 g, 2.5 min, 4°). The supernatants were transferred to 1.5-ml microfuge tubes and mixed by mild vortexing with approximately 1.5 volumes of 0.5 M alamine 336 in freon (200). After about 30 sec, the neutralized samples were centrifuged (12,800 g, 1 min), and the clear, top layers of supernatants (about 75 μl) were saved and stored frozen. The supernatants (50-55 μl) were later analyzed by HPLC using the same pump and detection system as for purification of tritiated *Vinca* alkaloids (Section D) and isocratic elution on a Whatman partisil ion exchange column (10/25, SAX) with 0.25 M KH_2PO_4 and 0.5 M KCl, pH 4.5, at 1.5 ml/min at room temperature (134). Nucleotide peaks were identified by comparison of retention time of chromatographed standards.

To assess the effect of depletion of cellular ATP on uptake of [^{3}H]VLB, exponentially proliferating cells were collected by centrifugation (120 g, 8 min) and resuspended (2 x 10^5 cells/ml) in "azide medium" containing 50 nM [^{3}H]VLB. Azide medium consisted of glucose-free RPMI 1640 supplemented with 15% dialyzed FBS and 10 mM sodium azide. After incubating cultures at 37° for 4 hr, triplicate 1-ml samples were removed for determination of cell-associated radioactivity as described in

Section G to obtain time-zero values for time courses of VLB release. The remainder of each culture was then centrifuged (120 g, 8 min), and VLB release was initiated by resuspending each pellet in drug-free azide medium at the same cell concentration as before centrifugation. The resulting cell suspensions were incubated at 37°, and, at graded time intervals thereafter, triplicate 1-ml samples were removed and cell-associated radioactivity was determined as described in Section G.

I. Materials

[^{3}H]VCR and [^{3}H]VLB were supplied by Moravek Biochemicals (Brea, CA). Vincristine sulphate and vinblastine sulphate were provided by Eli Lilly and Co., Indianapolis, IN, or were purchased from Sigma Biochemicals Co., St Louis, MO. Mithramycin was also purchased from Sigma Biochemicals Co. Materials for buffers (NaCl and HPLC grade KH_2PO_4) and HPLC grade methanol were supplied by Fisher Scientific, Fair Lawn, NJ. Tissue culture materials were purchased from Grand Island Biological Co., Burlington, Ont. Gentamicin was purchased from Schering Corporation, Kenilworth, NJ, and Hepes buffer from Research Organics, Inc., Cleveland, OH. Agar Noble is a product of DIFCO Laboratories, Detroit, MI. Microfuge tubes and micropipette tips were purchased from Bio-Rad Laboratories, Mississauga, Ont., and Triton X-100 was supplied by J. T. Baker Chemical Co., Phillipsburg, NJ.

V. Biological Effects of the Vinca Alkaloids on HL-60/C1 Cells

A. Introduction

Although there have been numerous studies of the antiproliferative and cytotoxic effects of the *Vinca* alkaloids against cultured cells, there are relatively few in which the effects of VCR and VLB have been compared directly at pharmacologically relevant concentrations. There are isolated reports of differences in cytotoxic activity of VCR and VLB against neoplastic cells in culture and transplantable tumors in mice (135), but at the time this study was initiated, it was not clear if the differences in activity observed in clinical use of VCR and VLB would be reflected by differential activity at the cellular level. The similarities in chemical structures (4) and in biochemical action against tubulin (25, 26) suggested that VCR and VLB would exhibit similar activities against proliferating cells.

Pharmacokinetic studies in humans have established that up to 95% of injected VCR or VLB is distributed into tissues within several minutes following injection (16), and the serum concentrations of VCR and VLB drop rapidly during the first few hr after injection (11). Thus, cells *in vivo* experience drug exposures in which extracellular drug concentrations are initially high (100-500 nM) for a short time (<0.5 hr), and then after a period of rapid decline, are low (1-10 nM) for an extended period (48-72 hr).

In a preliminary work (13), which was undertaken to determine if VCR and VLB exhibited differential activity against cultured cells, the early phase of *in vivo* drug exposure was modeled by subjecting cultured cells to pulse exposures of relatively high concentrations of VCR or VLB followed by culture in drug-free medium. The extended phase of drug exposure was modeled by subjecting cells to continuous exposures to low concentrations. Differential toxicity was seen after a short exposure interval, but not during continuous exposure, and the antiproliferative

and cytotoxic effects of VCR against several cell lines were significantly greater than those of VLB. In addition, there was no relationship between *in vivo* sensitivity of tissues of origin of cell lines and *in vitro* sensitivity to VCR and VLB. For example, HL-60 cells, which were originally included because it was thought they might reflect the selective hematopoietic toxicity characteristic of VLB *in vivo*, were equally sensitive to VCR and VLB during continuous exposures and much more sensitive to VCR than to VLB after short exposures.

HL-60 cells were selected to further study differential toxicity of the *Vinca* alkaloids, and results of biological studies of the effects of VCR and VLB against the clonal derivative, HL-60/C1, are presented in this chapter. As was earlier reported (13) for the parental line, VCR and VLB were equitoxic against HL-60/C1 cells during continuous exposures, and VCR was more toxic than VLB following 4-hr exposures. The possibility was considered that VCR and VLB might inhibit proliferation differently after short exposures. The primary intracellular target of the *Vinca* alkaloids is microtubule protein (25, 26), and there is considerable evidence that cytotoxicity results from inhibition of mitotic spindle formation (21, 22, 23, 24). The effects of continuous and 4-hr exposures of HL-60/C1 cells to VCR and VLB on DNA distributions were assessed by flow cytometry to determine if mitotic arrest was associated with cytotoxicity. Conditions were selected from proliferation experiments such that DNA distributions were obtained from cells that had been exposed to concentrations of VCR and VLB that inhibited proliferation rates to the same extent. Similar DNA distributions were obtained after exposure to equitoxic levels of drug, suggesting that VCR and VLB acted by the same mechanism, and that differential activity after 4-hr exposures was related to other factors.

B. Results

The effects of VCR and VLB on proliferation of HL-60/C1 cells during continuous exposures were virtually identical. Figure 4 is representative of the growth curves obtained when HL-60/C1 cells were exposed to graded concentrations of VCR or VLB (4-12 nM). Proliferation rates, obtained in 2 such experiments, are plotted as a function of extracellular concentrations of either drug in Figure 5, where it is apparent that the concentration-effect relationships for the 2 drugs were superimposable. The concentrations of VCR and VLB that inhibited proliferation rates by 50% (IC_{50} values) were 7.6 nM and 8.1 nM, respectively. During continuous exposures to either VCR or VLB, proliferation rates progressively decreased with increasing drug concentrations. The effect was immediate, and, at higher concentrations, did not diminish with time. At no concentration did proliferation rates recover to those of untreated cultures.

The dose-response relationships obtained for inhibition of proliferation of HL-60/C1 cells after short exposures to VCR and VLB were significantly different from those after continuous exposures. It is apparent from the results of a large series of experiments (Figure 5) that HL-60/C1 cells were much more sensitive to VCR than to VLB when subjected to 4-hr exposures, followed by culture in drug-free medium. The IC_{50} values for inhibition of proliferation by VCR and VLB were 41 nM and 1.1 μM, respectively. Differential activity of VCR and VLB after 4-hr exposures was also seen when the reproductive viability of drug-treated cells was assessed by assay of colony-formation in soft agar (Figure 6). The IC_{50} values for inhibition of proliferation rates and colony-forming ability by VCR and VLB obtained from a large series of experiments like those of Figures 4, 5, and 6 are summarized in Table 7. Although the IC_{50} for inhibition of colony-formation was lower than that for inhibition of proliferation following a 4-hr exposure to VCR, the opposite was true of 4-hr exposures to VLB. This is possibly a result of

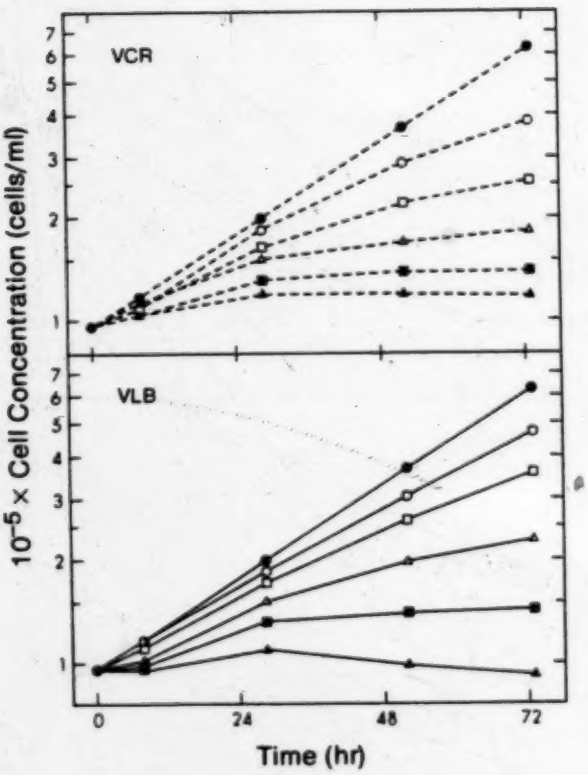


Figure 4. Effects of continuous exposures to VCR and VLB on proliferation of HL-60/C1 cells. Drug exposures were initiated by mixing suspensions of exponentially proliferating HL-60/C1 cells (2 x 10^5 cells/ml) with equal volumes of growth medium containing drug at twice the concentration to be tested, as described in Materials and Methods (Section A). The resulting cultures were incubated at 37° in 5% CO_2 in air, and cell concentrations were determined at the times indicated using a Coulter electronic particle counter. Concentrations of VCR and VLB were: ●, 0 nM; ○, 4 nM; □, 6 nM; △, 8 nM; ■, 10 nM; ▲, 12 nM.

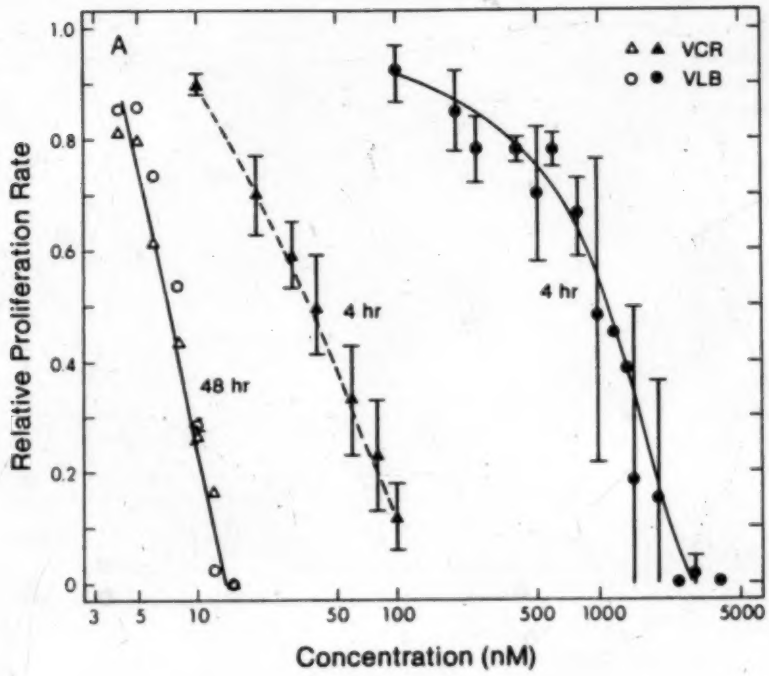


Figure 5. Effects of 48-hr and 4-hr exposures to VCR and VLB on rates of proliferation of HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1 x 10^5 cells/ml) in growth medium containing VCR or VLB at the concentrations indicated. Drug exposures were either continuous for 48 hr or for 4 hr, followed by centrifugation (120 g, 8 min), resuspension of cells in drug-free medium, and incubation at 37° for an additional 48 hr. Proliferation rates were determined from growth curves obtained as described for Figure 4 during the 48-hr period of drug exposure (48 hr) or during the 48-hr period of culture in drug free medium (4 hr). The population doubling time of untreated cultures was 24 hr. The proliferation rates (number of doublings of cell populations in 48 hr) of drug-treated cultures are expressed as fractions of values obtained for untreated cultures. *Points with bars*, means (± S.D.) of 2-5 experiments (including those of Figure 1); *points without bars*, results from a single experiment.

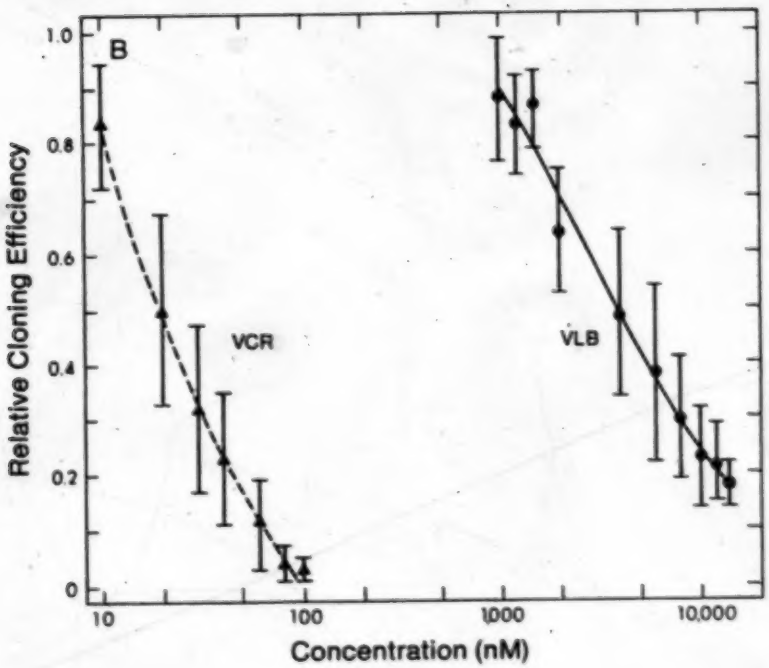


Figure 6. Effects of 4-hr exposures to VCR or VLB on colony-forming ability of HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1 x 10^5 cells/ml) in growth medium containing VCR or VLB at the concentrations indicated. After 4 hr, cultures were centrifuged (120 g, 8 min), and cells were resuspended in drug-free medium and cultured in soft agar as described in Materials and Methods (Section A). The absolute cloning efficiency (mean ± S.D., n=8 experiments) of untreated cells was 53 ± 5%. The cloning efficiencies of drug-treated cells are expressed as fractions of values obtained for untreated cultures. *Points with bars*, means (± S.D.) of 3 (VCR) and 5 (VLB) experiments.

Table 7

IC_{50} Values of VCR and VLB for Inhibition of Proliferation and Colony-Forming Ability of HL-60/C1 Cells

Assay	Length of Exposure (hr)	IC_{50} Values (nM)	
		VCR	VLB
Proliferation Rate	48	7.6(2)	8.1(2)
Proliferation Rate	4	41±9(5)	1100±430(5)
Cloning Efficiency	4	21±6(3)	3700±900(4)

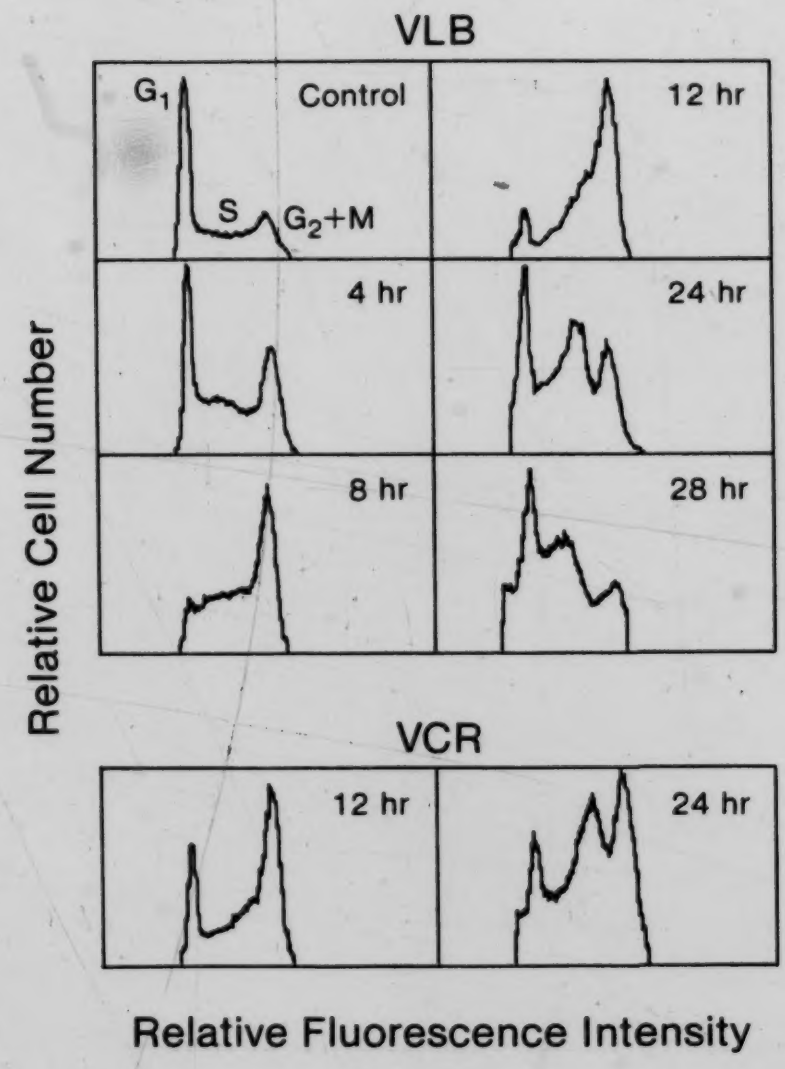
Data are from the experiments of Figures 5 (proliferation rates) and 6 (cloning efficiency). For proliferation experiments, the IC_{50} value is the concentration of drug that reduced proliferation rates to 50% of that of untreated cultures, and, for cloning experiments, the concentration that reduced cloning efficiencies to 50% of that of untreated cultures. Values are means (± S.D.) of the number of experiments indicated in parentheses.

re-equilibration of cell-associated VLB with the medium after resuspension of cells, which, in assays of proliferation, resulted in continuous exposure of cells to an extracellular VLB concentration on the order of 10 nM. This phenomenon is described further in Chapter VII (see Figure 22).

The effects of continuous exposures to VCR or VLB on progression of HL-60/C1 cells through the cell cycle were assessed by evaluating DNA distributions of drug-treated cultures at various times after drug exposures were initiated. In the experiments of Figure 7, cells were exposed to VLB or VCR at concentrations at or near IC_{50} values, and DNA distributions were obtained at graded time intervals by analysis of relative fluorescence intensity of mithramycin-stained cells by flow cytometry. For VLB, there were more cells in the S and G_2-M regions of DNA distributions and fewer cells in the G_1 region after 4 hr, and after 8 hr, the G_1 peak had almost completely disappeared. By 12 hr, the majority of cells had accumulated in the G_2-M peak, indicating a block in cell-cycle progression at or near mitosis. By 28 hr, cells that had managed to succesfully complete mitosis after being arrested in G_2-M at 12 hr were distributed in the G_1 and early S regions of DNA distributions. The DNA distributions obtained at 12 and 24 hr when HL-60/C1 cells were exposed to VCR were similar to those obtained at these times during exposures to VLB. Thus, HL-60/C1 cells accumulated in the G_2-M phase of the cell cycle during continuous exposures to either VCR or VLB.

In the experiments of Figure 7, the small peak visible in the G_1 region at 12 hr in DNA distributions of VLB-treated cells may represent a small synchronous fraction of cells that had escaped the G_2-M block. This population of cells had apparently moved into S phase by 24 hr and to G_2-M by 28 hr. Although a similar pattern was observed in cells exposed to VCR, the relative proportion of apparently synchronous cells was greater than that of VLB-treated cells.

Figure 7. Effects of continuous exposures of different lengths to partially inhibitory concentrations of VCR and VLB on DNA distributions of HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1 x 10^5 cells/ml) in growth medium containing 6 nM VLB or 10 nM VCR. At the times indicated, 15-ml portions of cultures were removed, fixed with 70% ethanol, stored at 4°, and prepared for analysis by staining with mithramycin as described in Materials and Methods (Section D). Fluorescence intensity of mithramycin-stained cells (representing relative DNA content) was determined with a Coulter EPICS V flourescence-activated cell sorter, and, for comparison, distributions have been normalized by the computer graphing program. Proliferation rates were determined as described for Figure 5 and are expressed as fractions of values obtained for untreated cultures. Relative proliferation rates for VLB and VCR-treated cultures were 0.51 and 0.62, respectively.


VLB
G1
Control
12 hr
S
G2+M
4 hr
24 hr
8 hr
28 hr
VCR
12 hr
24 hr
Relative Cell Number
Relative Fluorescence Intensity

The concentration-effect relationships between drug exposures and DNA distributions were explored in the experiments of Figures 8 and 9. After 12-hr exposures to VCR and VLB (Figure 8), cells had accumulated in the G_2-M peaks of DNA distributions. Accumulation was dose dependent, and, as the concentration of either drug was increased, the number of cells in the G_2-M peak also increased, with a concomitant loss of cells from the G_1 and S regions of DNA distributions. After 24-hr exposures to 2 and 4 nM VCR or VLB (Figure 9), the DNA distributions were similar to those obtained after 12-hr exposures. After 24-hr exposures to 6 nM VCR, a large fraction of the population was in the G_2-M peak, and after 24-hr exposures to 6 nM VLB, a large fraction of the population was in the early S-phase region of DNA distributions.

The effects of short (4-hr) exposures to VCR and VLB, followed by culture in drug-free medium, on DNA distributions of HL-60/C1 cells were assessed at drug concentrations that allowed comparison of cells that were inhibited by either drug to the same extent. It is apparent from the results presented in Figure 10 that the DNA distributions obtained 24 hr after initiation of drug exposure were identical for VCR and VLB-treated cultures with the same proliferation rates. For both drugs, a progressively smaller fraction of cells was present in the G_2-M peak as the degree of growth inhibition increased, suggesting that those cells that successfully underwent mitosis took increasingly longer to do so. Eight hr after transfer of VLB-exposed cells to drug-free medium (data not shown), there was a dose-dependent increase in the proportion of cells in the G_2-M peaks of DNA distributions, suggesting that short exposures also inhibited cells from progressing through the G_2-M phases of the cell cycle.

Figure 8. Effects of continuous exposures to equitoxic concentrations of VCR or VLB for 12 hr on DNA distributions of HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1×10^5 cells/ml) in growth medium containing VCR or VLB at the concentrations indicated. At 12 hr, 15-ml portions of cultures were removed, and processed for analysis by flow cytometry as described for Figure 7. Proliferation rates were determined as described for Figure 5 and are expressed as fractions of values obtained for untreated cultures. Relative proliferation rates of cultures exposed to 2, 4, 6, and 8 nM, respectively, were, for VCR 0.83, 0.52, 0.28, and 0.14, and, for VLB, 0.88, 0.54, 0.16, and 0.06.

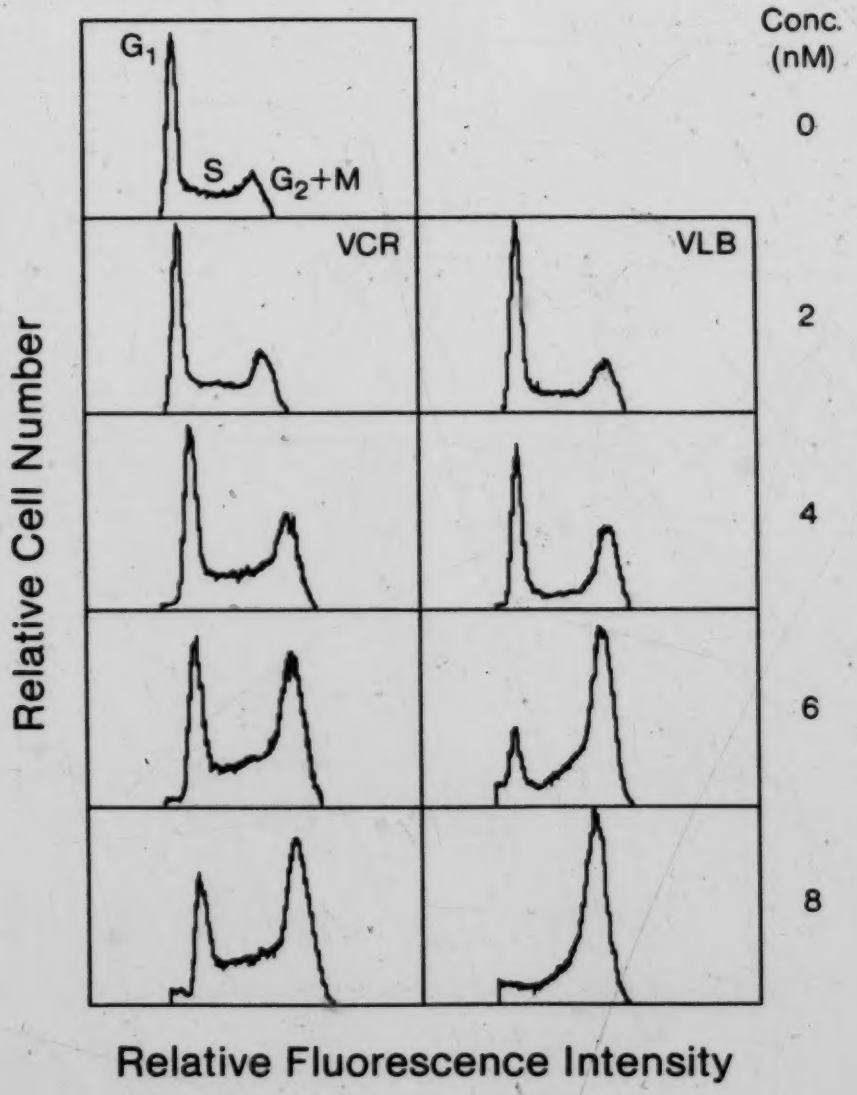

Conc.
(nM)
0
2
4
6
8
G1
S
G2+M
VCR
VLB
Relative Cell Number
Relative Fluorescence Intensity

Figure 9. Effects of continuous exposures of 24 hr to equitoxic concentrations of VCR or VLB on DNA distributions of HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1×10^5 cells/ml) in growth medium containing VCR or VLB at the concentrations indicated. At 24 hr, 15-ml portions of cultures were removed and processed for analysis by flow cytometry as described for Figure 7. Proliferation rates were determined as described for Figure 5 and are expressed as fractions of values obtained for untreated cultures. Relative proliferation rates of cultures exposed to 2, 4, and 6 nM, respectively, were, for VCR, 0.83, 0.52, and 0.28, and, for VLB, 0.88, 0.54, and 0.16.

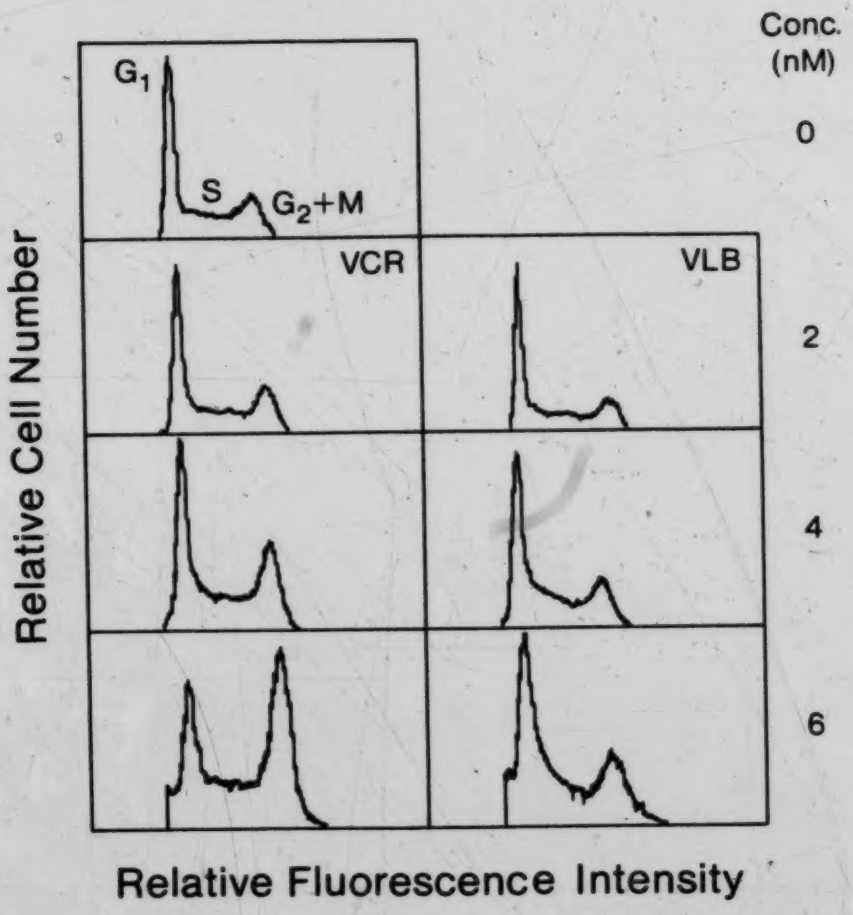
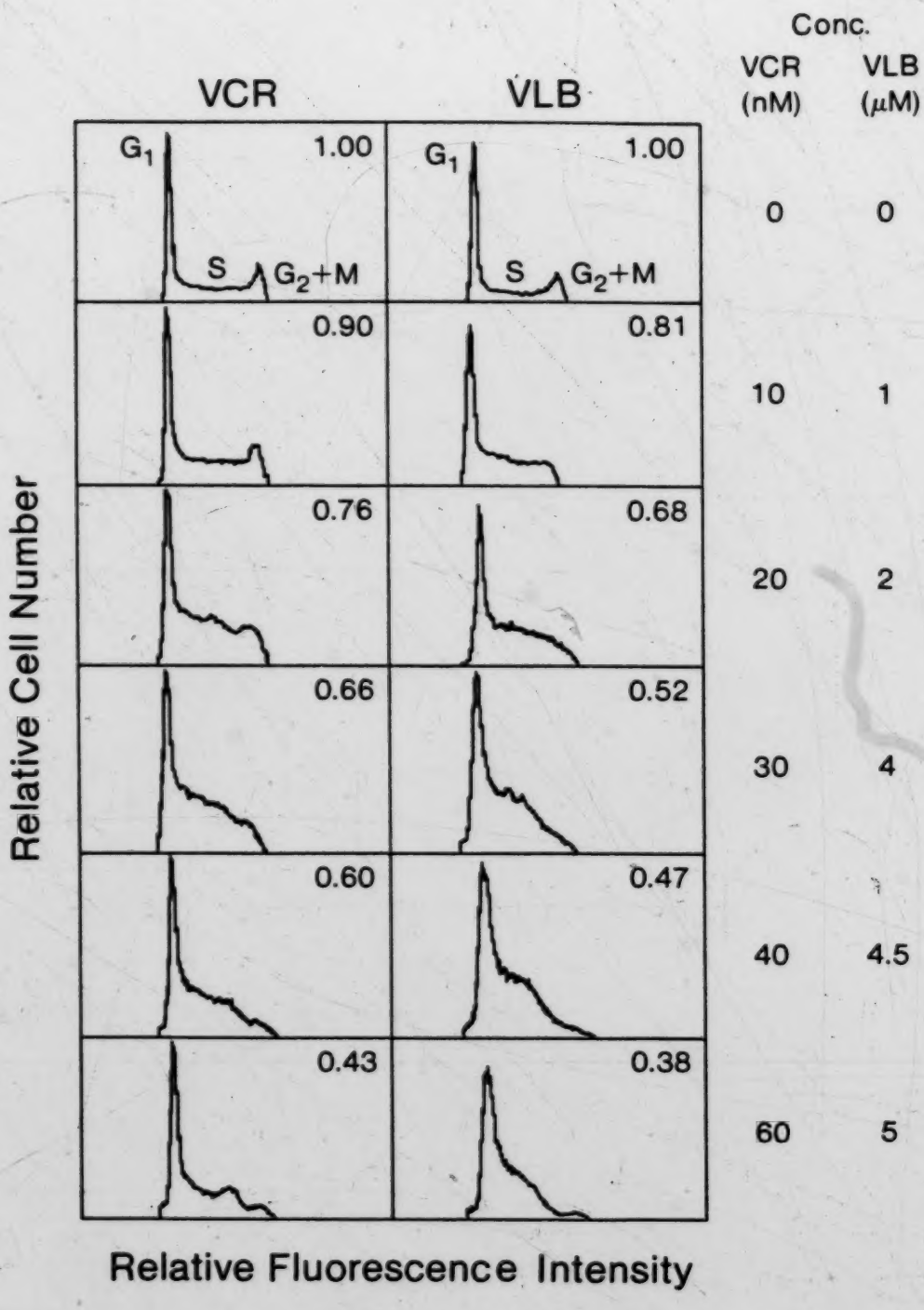

Conc. (nM)
0
2
4
6
G1
S
G2+M
VCR
VLB
Relative Cell Number
Relative Fluorescence Intensity

Figure 10. Effects of pulse exposures of 4 hr to equitoxic concentrations of VCR or VLB on DNA distributions of HL-60/C1 cells after culture for 20 hr in drug-free medium. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1×10^5 cells/ml) as described for Figure 4 in VCR or VLB at the concentration indicated. After 4 hr, cells were washed, resuspended in drug-free growth medium and incubated for 48 hr. At 24 hr, 15-ml portions of cultures were removed and processed for analysis by flow cytometry as described for Figure 7. Proliferation rates were determined as described for Figure 5 and are expressed as fractions of values obtained for untreated cultures. Relative proliferation rates are presented in the upper right-hand corner of the charts.


VCR
VLB
Conc.
VCR (nM)
VLB (μM)
G1
S
G2+M
1.00
1.00
0.90
0.81
0.76
0.68
0.66
0.52
0.60
0.47
0.43
0.38
0
0
10
1
20
2
30
4
40
4.5
60
5
Relative Cell Number
Relative Fluorescence Intensity

C. Summary

When cultured HL-60/C1 cells were subjected to continuous exposures to VCR and VLB at concentrations similar to those observed in human serum during the prolonged phase of drug clearance, proliferation rates were inhibited to the same degree, with IC_{50} values of about 8 nM. In contrast, differential toxicity was revealed when cells were subjected to short exposures to concentrations of VCR and VLB comparable to those during the early phase of drug clearance. Following 4-hr drug exposures, VCR was more toxic than VLB, with IC_{50} values, respectively, of 41 nM and 1.1 μM for inhibition of proliferation and 21 nM and 3.7 μM for inhibition of colony-formation. Analysis of DNA distributions of HL-60/C1 cells exposed VCR or VLB either continuously or for 4 hr, followed by transfer to drug-free medium, indicated that VCR and VLB inhibited proliferation by the same mechanism. With time, drug-treated cells accumulated with G_2-M contents of DNA during exposures to growth-inhibitory concentrations of either VCR or VLB, suggesting that both drugs acted primarily by inhibition of mitosis.

VI. Uptake and Metabolism of Vinca Alkaloids

A. Introduction

Uptake and release of VCR and VLB occur at different rates in platelets (27) and in cultured mouse leukemia L1210 cells (13). At equilibrium, platelets exposed to the same concentrations of either drug contained equal amounts of VCR or VLB. In studies of [^{3}H]VCR and [^{3}H]VLB uptake by cultured L1210 cells, incubations ($\leq$7 hr) were not of sufficient duration to achieve maximal uptake of both drugs. After 4-hr incubations, cell-associated VLB was several-fold greater than that of VCR, giving rise to the suggestion that cells have greater capacity for binding of VLB than VCR (13). The sensitivity of HL-60/C1 cells to the *Vinca* alkaloids is much like that of L1210 cells (13). Cellular content of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells was characterized during continuous exposures of up to 48 hr to determine if there were differences in rates of uptake and if, given sufficient time, the capacity for binding of the two drugs was the same. As well, loss of drug was measured from cells that were subjected to 24-hr exposures (equivalent to a single generation time) and cultured for a further 24 hr in drug-free medium. Uptake and release of VLB by HL-60/C1 cells was more rapid than that of VCR. Cellular capacity for uptake of either drug appeared to be the same, although, because of cellular deterioration during prolonged exposures, true steady-state levels of uptake were not achieved.

In the biological studies reported in Chapter V, the activities of VCR and VLB were compared by relating inhibition of proliferation rates and colony formation to concentrations of drug in culture fluids. Since the amounts of cell-associated drug were continuously changing (cellular content of both drugs increased to maximal values near 24 hr and then decreased), the activities of VCR and VLB were compared by relating inhibition of proliferation rates to intracellular drug content

over the period of evaluation. The "effective drug exposures" for VCR and VLB were determined by calculating *areas under curves* of plots of *cellular drug content versus time*. The relationships between inhibition of proliferation and effective drug exposures were the same for VCR and VLB.

In laboratory animals and humans, VLB is degraded to a greater extent than VCR; degradation occurs in the liver (28, 29, 30, 31, 32, 33) and possibly also in plasma (30, 34). VLB does not appear to be metabolized by cultured human leukemic lymphoblasts or by growth medium (35). Since metabolism may have altered extracellular concentrations during drug exposures, HL-60/C1 cells were cultured with [^{3}H]VCR or [^{3}H]VLB, and extracts of cells and culture fluids were analyzed by HPLC for evidence of possible metabolites. During 24-hr exposures, neither VCR nor VLB were metabolized significantly by cells, and degradation and/or metabolism by culture fluids was minor.

VCR and VLB both adsorb significantly to serum proteins. At drug concentrations of 1-100 μM, about 50% of VCR or VLB present in native or lipid-free human serum binds to serum proteins (136). The major alkaloid-binding protein of human serum, α_1-acid glycoprotein, apparently has 2 *Vinca* binding sites, with Ka values for VLB of 9.4 x 10^6 and 0.1 x 10^6 M^{-1} (36). Binding of [^{3}H]VCR and [^{3}H]VLB to the proteins of fetal bovine serum was assessed to determine if there were differences in the concentrations of free drug to which HL-60/C1 cells were exposed. Although VCR and VLB were bound to serum proteins of culture fluids to a significant extent, the fraction bound over a wide range (0.05 - 3 μM) of concentrations was similar for both drugs.

Because the higher of the 2 pKa values[1] of the basic nitrogens of VCR and VLB is the same as the pH of the growth medium, slight changes in pH of culture fluids during drug exposures could significantly alter the proportion of ionized

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[1]pKa values (38) are: VCR, 7.4 and 5.0; VLB, 7.4 and 5.4.

Vinca molecules. The effects of pH on binding of [^{3}H]VCR and [^{3}H]VLB to proteins of fetal bovine serum and on cellular uptake of [^{3}H]VCR and [^{3}H]VLB were assessed to determine if either of these processes was affected by ionization of drug. With increasing pH (from 5.5 to 8.0), the amount of [^{3}H]VCR bound to serum proteins changed little, whereas the amount of VLB bound increased 2-fold. Although uptake of both drugs by HL-60/C1 cells increased with increasing pH, the pH-dependence of VLB uptake was greater than that of VCR. The pH-dependence of *Vinca* uptake was demonstrated previously with L1210 cells (13).

B. Results

The method used to quantitate cellular drug content involved assay of cell-associated radioactivity after drug-treated samples were removed from cultures and washed with ice-cold physiological saline. The experiments of Figures 11 and 12 were conducted to establish that the washing procedures used to terminate uptake reactions did not greatly reduce cell-associated radioactivity. When HL-60/C1 cells were exposed to 50 nM [^{3}H]VCR or [^{3}H]VLB for 4 hr (Figure 11) or 8 hr (Figure 12), cell-associated radioactivity was not altered significantly by washing cells up to 4 times with ice-cold saline. Since the number of cells per pellet declined during multiple washes, one wash was used routinely in subsequent determinations.

Cellular uptake of [^{3}H]VCR and [^{3}H]VLB was examined over a range of concentrations that included the IC_{50} values (8 nM) for inhibition of proliferation during continuous exposures. Figure 13 illustrates the results of experiments in which HL-60/C1 cells were exposed to drug either continuously for 48 hr, or for 24 hr followed by resuspension in drug-free medium. At each concentration, more VLB had entered cells within the first 1-2 hr than had VCR, indicating that cellular uptake of VLB was more rapid than that of VCR. The cellular content of VLB was greater than that of VCR during the first 24 hr and less during the second 24

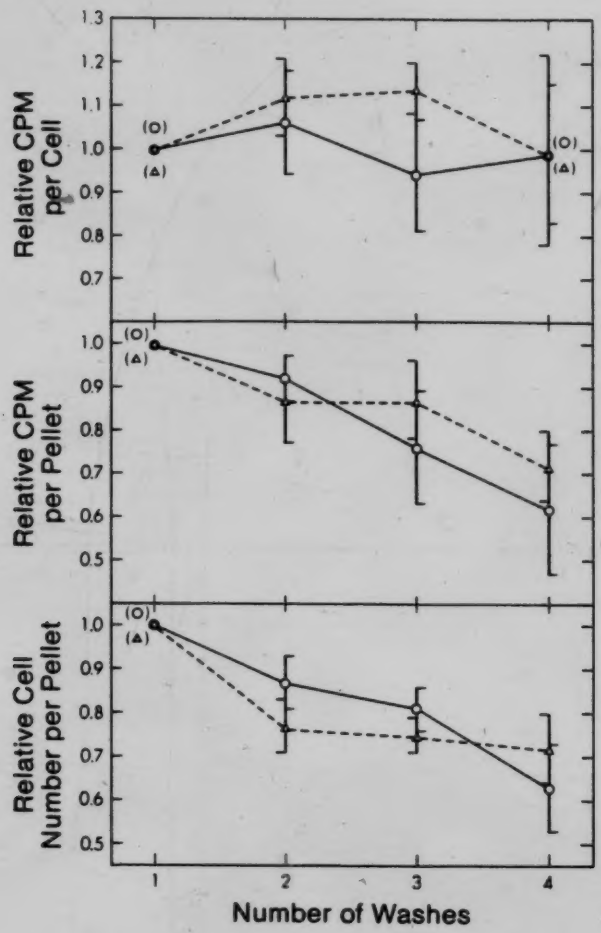

Figure 11. Effect of multiple washes on cell-associated radioactivity after 4-hr exposures to [^{3}H]VCR and [^{3}H]VLB. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in either 50 nM [^{3}H]VCR (Δ) or 50 nM [^{3}H]VLB (o) in growth medium containing 4 mM Hepes, pH 7.4. After 4 hr at 37°, 1-ml portions of cell suspensions were removed, centrifuged (12,800 g, 1 min), and washed the number of times indicated by resuspension of cells in 1 ml ice-cold saline, followed by centrifugation (12,800 g, 1 min). The radioactive content and number of cells were determined as described in Materials and Methods (Section G). The values (mean ± S.D., n=3) obtained after successive washes are presented as fractions of the values obtained after 1 wash.

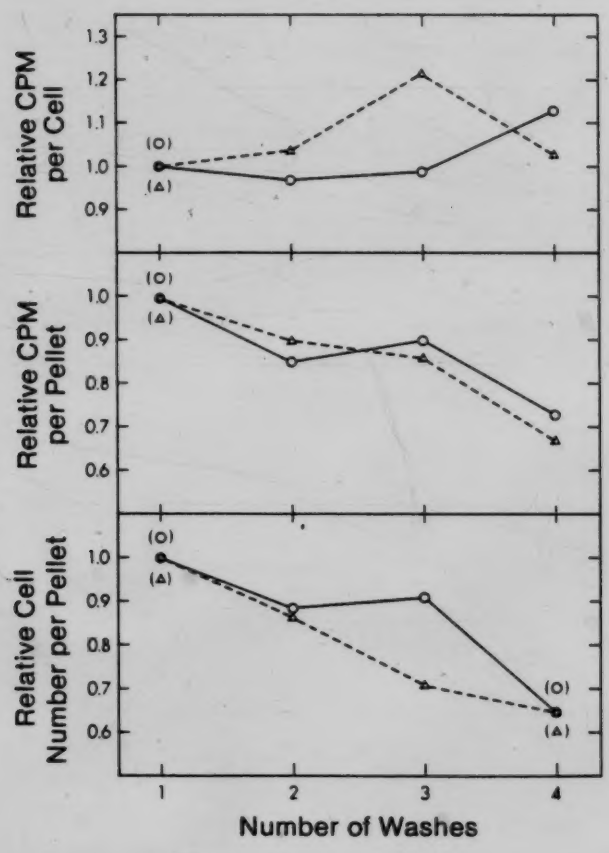

Figure 12. Effect of multiple washes on cell-associated radioactivity after 8-hr exposures to [^{3}H]VCR or [^{3}H]VLB. Hl-60/C1 cells were exposed to 50 nM [^{3}H]VCR (Δ) or [^{3}H]VLB (o) for 8 hr and cell-associated radioactivity was determined after each of 4 washes with ice-cold saline as described in Figure 11. The values obtained after successive washes are presented as fractions of the values obtained after 1 wash. Individual data *points* represent the results of a single experiment.

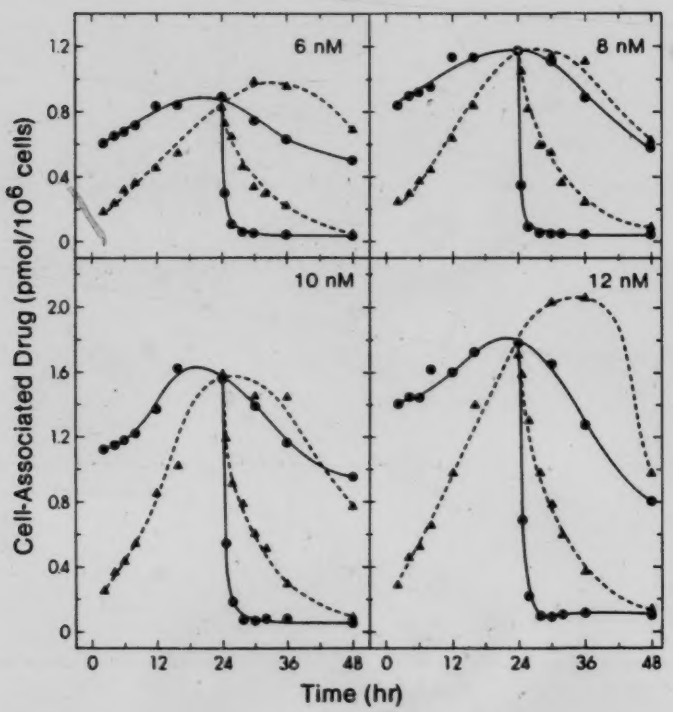

Figure 13. Time courses of uptake and release of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1 x 10^5 cells/ml) in either [^{3}H]VCR (▲) or [^{3}H]VLB (●) at the concentrations indicated in growth medium containing 4 mM Hepes, pH 7.4. Drug exposures were either continuous for 48 hr or for 24 hr, followed by culture in drug-free medium for 24 hr. In the latter case, cells were collected by centrifugation (120 g, 8 min) and resuspended in drug-free growth medium (approx. 1 x 10^5 cells/ml). All incubations were performed at 37°. Cell-associated radioactivity was determined at graded time intervals as described in Materials and Methods (Section G), and values (2 determinations per condition) presented were calculated assuming that cell-associated radioactivity was equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB.

hr, and, at 24 hr, the amount of cell-associated radioactivity, presumably present as VCR or VLB, was the same for each of the 4 concentrations tested (Table 8). As well, the apparent intracellular concentrations of VCR and VLB were significantly greater than extracellular concentrations, suggesting binding of drug to cellular components. When cells were washed at 24 hr and resuspended in drug-free medium, although VLB was released more rapidly than VCR, the amount of either drug remaining in cells at 48 hr was the same.

It is apparent from the results of Figure 13 that VCR and VLB were lost from cells after 24 hr even during continuous exposure to radiolabelled drug. The decline in drug content did not appear to be due to "dilution" of radioactivity by the presence of newly formed cells since cell numbers declined, although only slightly, during 24-hr exposures (Table 9). Intracellular binding sites may have decreased during extended exposures, or drug may have been extruded by cells, perhaps by vesiculation of drug-containing plasma membrane, a phenomenon that has been observed in VLB-treated cells (64).

"Effective drug exposures" were determined for the experiments of Figure 13 by calculating the *areas under curves* for 48 hr continuous exposures or for 24-hr exposures, followed by a 24-hr culture in drug-free medium. It is apparent from the relationships between relative proliferation rates and "effective drug exposures" (Figure 14) that VCR and VLB were equitoxic against HL-60/C1 cells. For both 48-hr continuous exposures and 24-hr exposures, followed by culture for 24 hr in drug-free medium, the curves obtained for these relationships were superimposable.

To determine if there was metabolism of VCR or VLB during continuous exposures, HL-60/C1 cells were cultured in the presence of growth-inhibitory levels of [^{3}H]VCR or [^{3}H]VLB, and the amounts of radiolabelled drug and "metabolites" were quantitated by HPLC. In these experiments, >99% of pellet radioactivity was recovered by the extraction procedure, and spontaneous decomposition of [^{3}H]VCR

Table 8

Cell-Associated Drug after 24-hr Continuous Exposures

Concentration (nM)	pmol/10^6 cells		Accumulation [drug]in/[drug]out	
	VCR	VLB	VCR	VLB
6	0.828	0.890	99	106
8	1.13±0.11[1]	1.11±0.23[1]	101	99
10	1.60	1.56	114	111
12	1.72	1.78	102	106

[1]mean (± S.D., n=4 experiments, including those of Figure 13).

Unless otherwise noted, data are from the experiments of Figure 13. The values presented were calculated assuming that cell-associated radioactivity was equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB. Accumulation is presented as the ratio of "apparent intracellular concentration" to extracellular concentration of VCR and VLB at 24 hr. Apparent intracellular concentrations were calculated using average cell volumes obtained with a Coulter channelyzer as described in Materials and Methods (Section B).

Table 9

Cell Recovery During Uptake Experiments of Figure 13

Drug	Conc.	Cell Number (10^5/ml)[1]			
	(nM)	24 hr	30 hr	36 hr	48 hr
VCR	6[2]	1.31	1.22	1.25	1.04
	8[2]	1.12	1.13	1.01	0.90
	10	0.96	1.01	1.00	0.84
	12	1.13	1.00	0.92	0.92
VLB	6[2]	1.09	1.15	1.04	0.88
	8[2]	1.06	0.96	0.88	0.77
	10	1.00	0.96	0.85	0.79
	12	0.99	0.97	0.86	0.98

[1]Values are from the experiments of Figure 13.

[2]Although cell concentrations increased in cultures during exposure to drug at these concentrations, cell recoveries in uptake experiments declined between 24 and 48 hr because of loss of cells during the washing procedure.

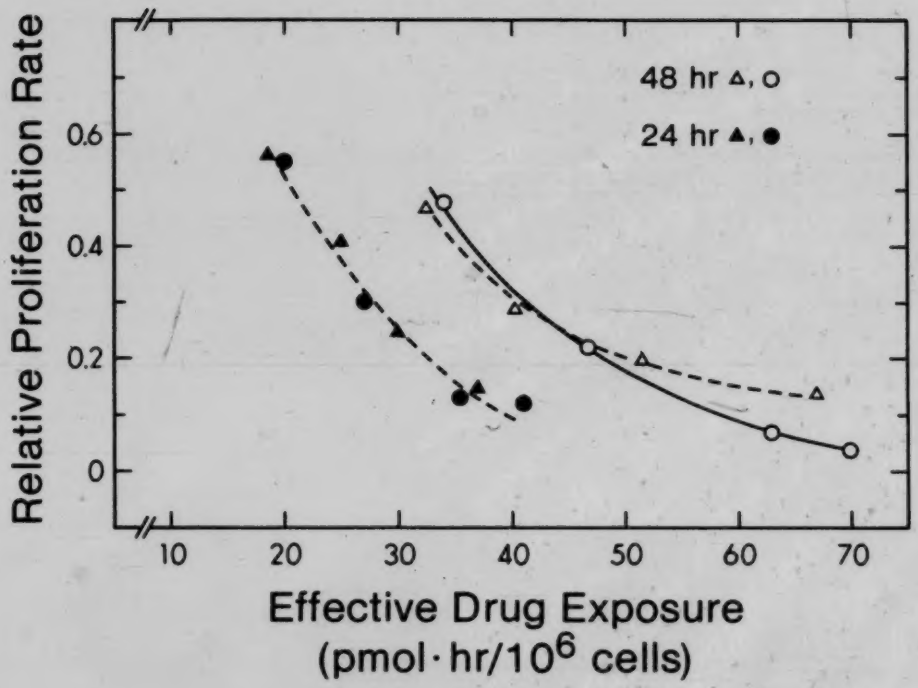

Figure 14. Proliferation rate of HL-60/C1 cells as a function of effective drug exposure. Data include the results of the experiments of Figures 4 and 13. The "effective drug exposures" for VCR (△, ▲) and VLB (○, ●) were determined from the *areas under curves* of plots of *cellular drug content* (pmol/10^6 cells) *versus time over 48 hr*. Drug exposures were continuous for 48 hr or for 24 hr, followed by culture for 24 hr in drug-free medium. Relative proliferation rates of drug-treated cultures are expressed as fractions of absolute proliferation rates (number of population doublings in 48 hr) of untreated cultures. The population doubling time of untreated cultures was 24 hr. Values are presented as the means of 2 experiments.

and [^{3}H]VLB did not occur during extraction or HPLC analysis. The small variations in retention times of VCR and VLB in the different profiles are the result of minor changes in solvent gradients. Figures 15 and 16 demonstrate elution profiles of radioactivity extracted from cells and culture fluids after 24 hr incubation with either [^{3}H]VCR or [^{3}H]VLB. The relative proportions of radioactivity present in extracts as unaltered drug are presented in Table 10. After 24 hr, all of cell-associated radioactivity in VCR-exposed cells and almost all in VLB-exposed cells was recovered as unchanged drug. Most of the radioactivity of culture fluids was recovered as unchanged VCR or VLB. Thus, there was little, if any, metabolism of either drug by HL-60/C1 cells, and the small change in concentration of radiolabelled drug in culture fluids, which was also seen in growth medium without cells, may have resulted from spontaneous degradation.

Binding of VCR or VLB to the proteins present in fetal bovine serum of culture fluids may have altered the extracellular concentration of drug, and, if either drug was bound to a greater or lesser extent, may have contributed to differences in activity. Binding of [^{3}H]VCR and [^{3}H]VLB to serum proteins was assayed by equilibrium dialysis over a wide range of drug concentrations (Table 11). Under the conditions of most uptake and proliferation experiments (pH 7.5 and 15% FBS), about 26% of VCR and 24% of VLB was bound to serum proteins. The binding of both drugs decreased when serum concentrations were lowered from 15 to 2%, and at 15% serum, binding was not saturated at the highest concentration of VCR (400 nM) or VLB (3 μM) tested.

The effects of fetal bovine serum on cellular uptake of [^{3}H]VCR and [^{3}H]VLB are illustrated in Figure 17. Cellular content of both drugs following 4-hr exposures decreased as serum concentrations were raised from 2 to 15%. Since there was less free drug present at higher serum concentrations, these results suggest that free, and not bound, drug entered cells and that binding of *Vinca* alkaloids to

Figure 15. HPLC analysis of HL-60/C1 cultures after 24-hr exposures to [^{3}H]VCR. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in the absence (Panel A) or presence (Panels B and C) of 20 nM [^{3}H]VCR in growth medium containing Hepes, pH 7.4. Growth medium (without cells) containing 20 nM [^{3}H]VCR was also evaluated (not shown). After incubation at 37° for 24 hr, cultures were centrifuged (120 g, 8 min), and cell pellets and culture fluids were processed for analysis by HPLC as described in Materials and Methods (Section F). Before analysis of extracts by HPLC, 5 pmol of non-radioactive VCR were added as carrier. Individual extracts (200 µl) usually contained 10^4 - 5 x 10^4 cpm. Presented are elution profiles of absorbance at 259 nm (solid lines) and radioactivity (cpm/fraction; dotted lines) of extracts obtained from: cells incubated in the absence of drug and to which [^{3}H]VCR (20 pmol) was added (to washed pellets) just before beginning the extraction procedure, Panel A; cells incubated for 24 hr in the presence of [^{3}H]VCR, Panel B; culture fluids after 24-hr incubations of cells with [^{3}H]VCR, Panel C.

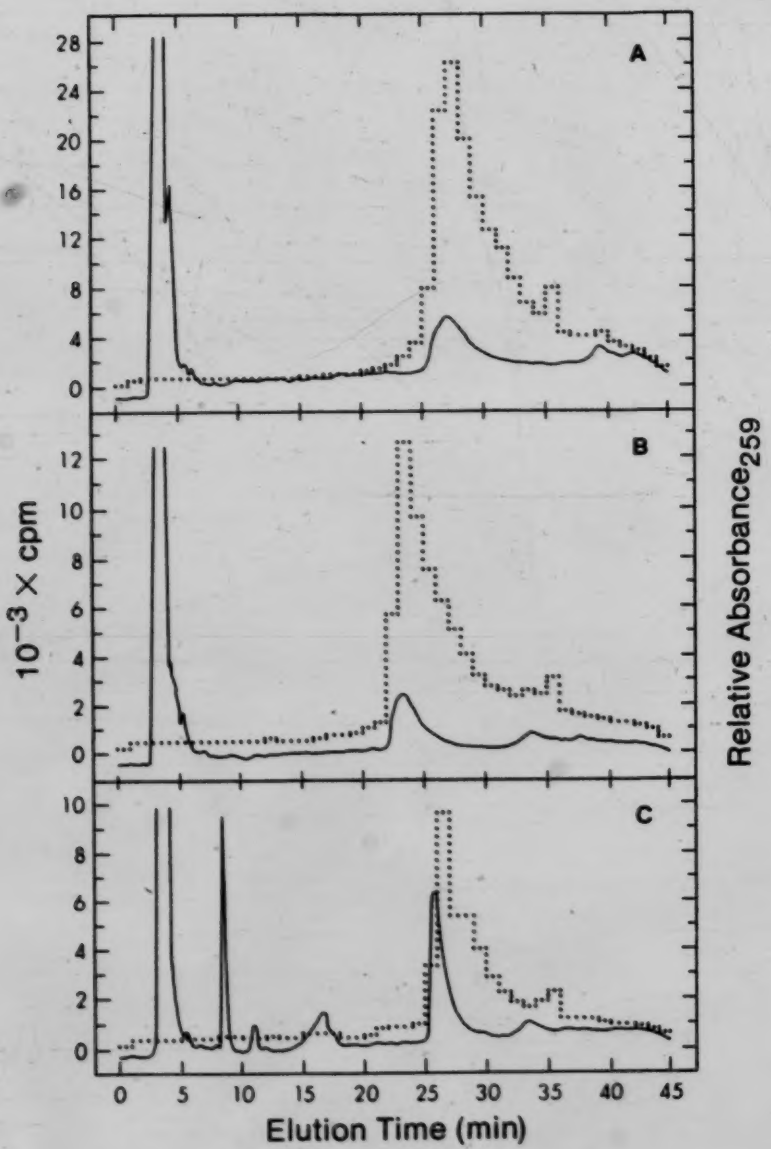
A
B
C
10^{-3} X cpm
Relative Absorbance$_{259}$
Elution Time (min)
0
5
10
15
20
25
30
35
40
45

Figure 16. HPLC analysis of HL-60/C1 cultures after 24-hr exposures to [^{3}H]VLB. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in the absence (Panel A) or presence (Panels B and C) of 20 nM [^{3}H]VLB in growth medium containing Hepes, pH 7.4. Growth medium (without cells) containing 20 nM [^{3}H]VLB was also evaluated (not shown). After incubation at 37° for 24 hr, cultures were centrifuged (120 g, 8 min), and cell pellets and culture fluids were processed for analysis by HPLC as described in Materials and Methods (Section F). Before analysis of extracts by HPLC, 5 pmol of non-radioactive VLB were added as carrier. Individual extracts (200 µl) usually contained 10^4 - 5 x 10^4 cpm. Presented are elution profiles of absorbance at 259 nm (solid lines) and radioactivity (cpm/fraction; dotted lines) of extracts obtained from: cells incubated in the absence of drug and to which [^{3}H]VLB (20 pmol) was added (to washed pellets) just before beginning the extraction procedure, Panel A; cells incubated for 24 hr in the presence of [^{3}H]VLB, Panel B; culture fluids after 24-hr incubations of cells with [^{3}H]VLB, Panel C.

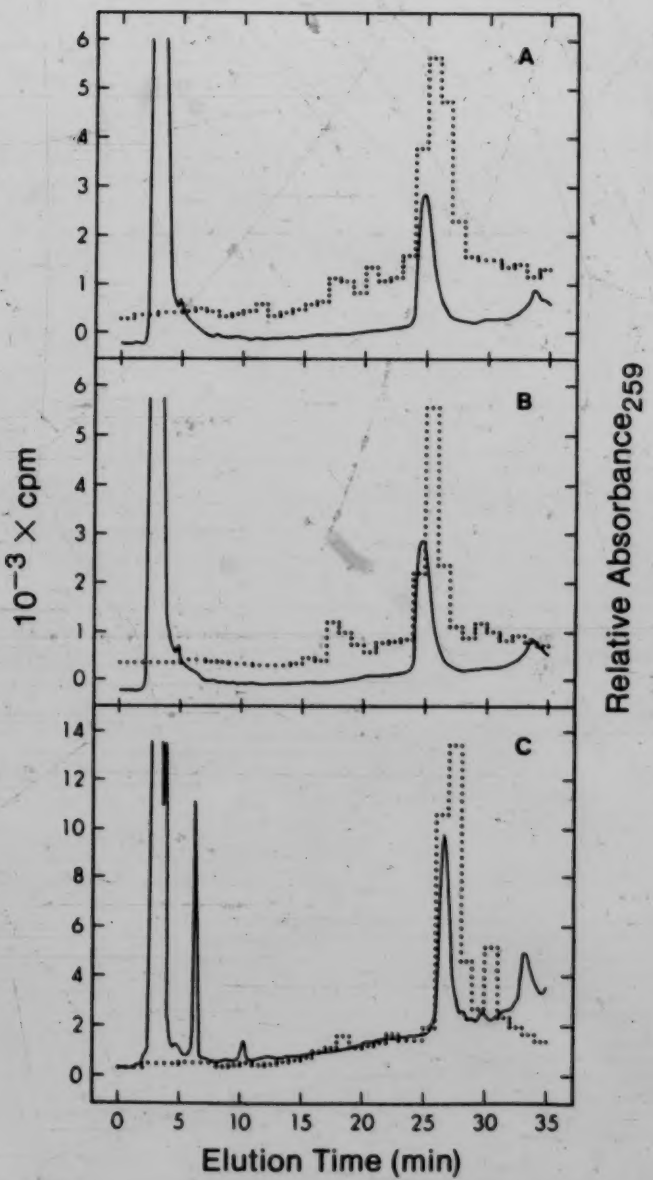
A
B
C
10^{-3} × cpm
Relative Absorbance$_{259}$
Elution Time (min)
0
5
10
15
20
25
30
35

Table 10

Recovery of [^{3}H]VCR and [^{3}H]VLB from HL-60/C1 Cultures After 24-hr Exposures

Sample	% of Radioactivity Present in:	
	VCR	VLB
Cells, 0 hr	>95	>99
Cells, 24 hr	>99	87-100[1]
Medium (without cells), 24 hr	84	88
Medium (with cells), 24 hr	83-93[1]	96

[1]Results of 2 determinations.

Data are from the experiments of Figures 15 and 16. The amount of radioactivity that co-eluted with authentic VCR or VLB, respectively, has been expressed as a percentage of the total radioactive content of extracts (10^4 - 5 x 10^4 cpm/200 μl).

Table 11. Binding of [^{3}H]VCR and [^{3}H]VLB to proteins present in fetal bovine serum as a function of (a) serum concentration, (b) pH, or (c) drug concentration was studied by equilibrium dialysis as described in Materials and Methods (Section E). Results of the 3 experimental procedures are summarized, and, for each, values (mean ± S.D.; number of determinations indicated in parentheses) for the amount of radioactivity bound, at equilibrium, to serum proteins are expressed as a percentage of total radioactivity (15,000-60,000 cpm) in assay mixtures.

Table 11

Binding of [^{3}H]VCR and [^{3}H]VLB

to Proteins of Fetal Bovine Serum

Drug Concentration (nM)	Serum Concentration (%)	pH	Amount Bound (%)	
			VCR	VLB
50	2	7.5	13±2(4)	10.3±2.8(4)
	5		22±2(4)	13.2±0.9(4)
	10		23±1(4)	20.2±3.1(4)
	15		26±3(5)	23.8±3.2(4)
50	15	5.5	29.3±0.8(3)	15.0±2.5(4)
		6	24.3±2.0(4)	20.5±0.8(3)
		6.5	27.9±2.6(3)	21.2±1.8(4)
		7	27.6±0.6(2)	22.6±1.2(4)
		7.5	26±3(5)	23.8±3.2(4)
		8	27.2±4.0(4)	24.5±1.4(4)
50	15	7.5	26±3(5)	23.8±3.2(4)
100			29.4±0.4(2)	
200			26±1(4)	24.2±0.5(2)
400			26±2(3)	
800				25.9±2.5(2)
1000				23.7±2.7(2)
3000				24.8±4.6(2)

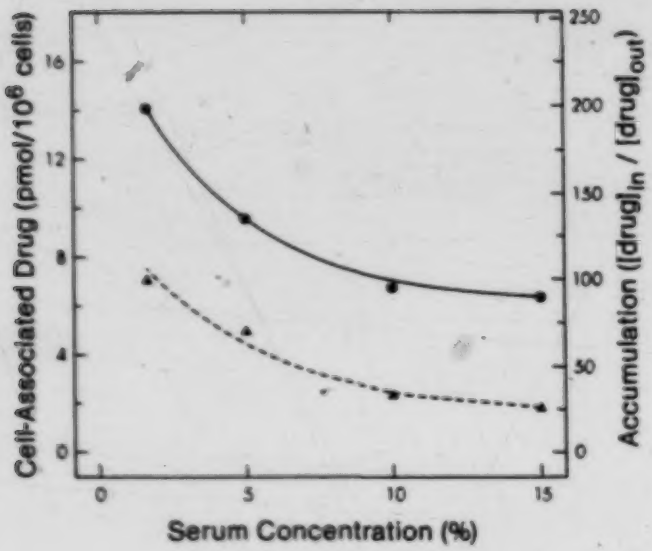

Figure 17. Effects of serum concentration on uptake of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in 50 nM [^{3}H]VCR (▲) or [^{3}H]VLB (●) in RPMI 1640 containing 4 mM Hepes buffer, pH 7.4, and supplemented with fetal bovine serum at the concentrations indicated. The resulting cultures were incubated at 37°, and after 4 hr, cell-associated radioactivity was determined as described in Materials and Methods (Section G). Values (mean, n=3) were calculated assuming that cell-associated radioactivity was equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB. Accumulation is presented as the ratio of "apparent intracellular concentration" to extracellular concentration at 4 hr. Apparent intracellular concentrations were calculated using average cell volumes obtained with a Coulter channelyzer as described in Materials and Methods (Section B). Results are representative of those obtained in 3 separate experiments.

serum proteins reduced drug effectiveness.

The effects of pH on uptake of VCR and VLB by HL-60/C1 cells were explored in the experiments of Figure 18. Although cellular uptake of both drugs increased with increasing pH, the profiles obtained were different in shape. VCR uptake increased gradually between pH 5.5 and 8.0, reaching a maximum at pH 8.0, whereas VLB uptake increased sharply, reaching a maximum at pH 7.5 and declining somewhat at pH 8.0. The difference in pH profiles may be related to the greater hydrophobicity of VLB than VCR in that, as the proportion of neutral molecules increased, there may have been greater partitioning of VLB, than VCR, in the hydrophobic phase of cell membranes and other hydrophobic cellular constituents. It is also possible that the charge environment of the cell surface changed with increasing pH, which may have altered hydrophobic and hydrophilic interactions between drug and cells.

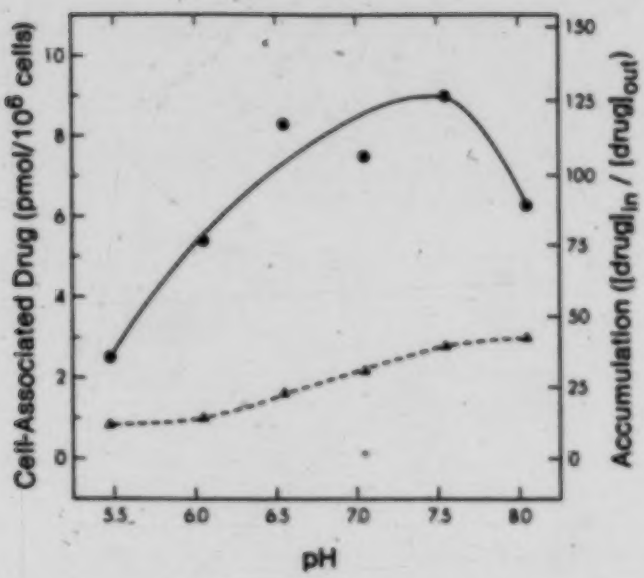

Figure 18. Effects of pH on uptake of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in 50 nM [^{3}H]VCR (▲) or [^{3}H]VLB (●) in growth medium containing 4 mM Hepes at the pH values indicated. The resulting cultures were incubated at 37°, and after 4 hr, cell-associated radioactivity was determined as described in Materials and Methods (Section G). Values (mean, n=3) were calculated assuming that cell-associated radioactivity was equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB. Accumulation is presented as the ratio of "apparent intracellular concentration" to extracellular concentration at 4 hr. Apparent intracellular concentrations were calculated using average cell volumes obtained with a Coulter channelyzer as the described in Materials and Methods (Section B). Results are representative of those obtained in 3 separate experiments.

C. Summary

During continuous exposures of HL-60/C1 cells to [^{3}H]VCR or [^{3}H]VLB under conditions of proliferation experiments, VLB entered cells more rapidly than VCR. Accumulation of both drugs was "concentrative" and maximal accumualtion was seen at or near 24 hr. At 24 hr, the cell contents of VCR and VLB were about the same, suggesting that HL-60/C1 cells have similar capacities for uptake of either drug. After 24 hr, in the continued presence of extracellular drug, cell-associated VCR and VLB declined, suggesting loss of *Vinca* binding sites, perhaps resulting from cell deterioration. When cells containing [^{3}H]VCR or [^{3}H]VLB were resuspended in drug-free growth medium after 24-hr exposures, VLB was lost from cells more rapidly than was VCR.

"Effective drug exposures" were determined by calculating *areas under curves* of plots of *cellular drug content versus time*. For 48-hr continuous exposures or for 24-hr exposures followed by 24-hr culture in drug-free medium, the relationships between "effective drug exposures" and inhibition of proliferation rates by VCR and VLB were the same. Thus, even though the intracellular content of either drug changed continuously with time, the net effect on proliferative capacity was the same for VCR and VLB.

There was little, if any, metabolism of VCR and VLB by HL-60/C1 cells during 24-hr continuous exposures. The small decreases in VCR and VLB in culture fluids may have been due to spontaneous degradation during 24-hr incubations.

Under cell culture conditions, about 25% of either drug was bound to the proteins of fetal bovine serum, and binding to serum proteins decreased at low serum concentrations. Uptake of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells was greatest at low serum concentrations, suggesting that free, and not protein-bound, drug entered cells.

Uptake of both [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells increased with increasing pH, suggesting, since both drugs were present primarily as neutral molecules at high pH, that either drug penetrates cells more readily when in the neutral state than when charged. The pH dependence of VLB uptake was greater than that of VCR. Thus, since VLB is more hydrophobic than VCR, the differences in cell contents of the two drugs may be due to greater hydrophobic interaction of VLB with cellular constituents.

VII. Uptake and Release of Vinca Alkaloids after 4-hr Exposures

A. Introduction

Although VCR was much more toxic to HL-60/C1 cells than VLB after short exposures, analysis of DNA distributions of cells treated for 4 hr with equitoxic concentrations suggested that VCR and VLB inhibited proliferation by similar mechanisms. Since differential activity after short-term exposures was apparently not due to different mechanisms, the possibility was considered that differences in cellular uptake of VCR and VLB were responsible for differential activity. Differences in rates of uptake and release of VCR and VLB have been previously observed (13, 27, 137). VLB is released more rapidly than VCR from rat platelets and lymphoma cells (27), and differential toxicity in L1210 leukemia cells, after transfer of *Vinca*-treated cells to drug-free medium, was attributed to the greater retention of VCR, than of VLB, by cells (13).

To determine if differences in cellular retention of drug were responsible for differential activity in HL-60/C1 cells, uptake and release of [^{3}H]VCR and [^{3}H]VLB were measured under conditions of 4-hr proliferation experiments. Initially, uptake over 4 hr was measured at 50 nM, a drug concentration near the IC_{50} value for VCR and much less than that for VLB. Much more VLB than VCR entered cells during these exposures, suggesting, since they apparently acted by similar mechanisms at equitoxic concentrations, interaction of VLB during 4-hr exposures with cellular components other than the cytotoxic target. Rates of release of [^{3}H]VCR and [^{3}H]VLB from cells were then determined after exposures for 4 hr to concentrations above and below the IC_{50} value for VCR toxicity, and for VLB, after exposures of cells to concentrations near the IC_{50} value for VLB toxicity. Rates of release of VCR and VLB were compared directly by exposing HL-60/C1 cells to "pairs" of concentrations of [^{3}H]VCR and [^{3}H]VLB such that cells contained the same amounts

of either drug at 4 hr. At all pairs of concentrations studied, the amount of cell-associated VLB at 4 hr was greater than that of VCR, and VLB was released more rapidly than VCR.

The "effective drug exposures" were calculated from plots of *areas under curves* of *cellular drug content versus time* during 4-hr exposures, followed by culture of cells in drug-free medium. The relationships between "effective drug exposures" and loss of viability, as measured by assay of colony formation, were quite different for VCR and VLB. Higher "effective drug exposures" were required for VLB, than for VCR, to achieve the same degree of growth inhibition.

B. Results

Measurements of uptake and release of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells subjected to 4-hr exposures to drug were conducted over a wide range of concentrations. Figure 19 demonstrates the results of uptake experiments near the IC_{50} value (21 nM) of VCR for inhibition of colony-forming ability. At each concentration, the amount of cell-associated VCR was less than that of VLB, and when cells were washed at 4 hr and resuspended in drug-free medium, VLB was released more rapidly than was VCR.

In the experiments of Figure 20, the time courses of uptake and release of [^{3}H]VCR and [^{3}H]VLB were examined in greater detail at 50 nM, a concentration slightly more than the IC_{50} value (41 nM) of VCR for inhibition of proliferation. The progress curve for cellular uptake of VLB was steeper than that of VCR, and at 4 hr, the amount of cell-associated VLB was more than 4-fold greater than that of VCR. Both drugs were "concentrated" by cells in that, by 4 hr, the ratios of apparent intracellular to extracellular drug concentration were, for VCR and VLB, respectively, 30 and 130. When cells were transferred to drug-free medium at 4 hr, VLB was released more rapidly than VCR. Within 1.5 hr, the release curves

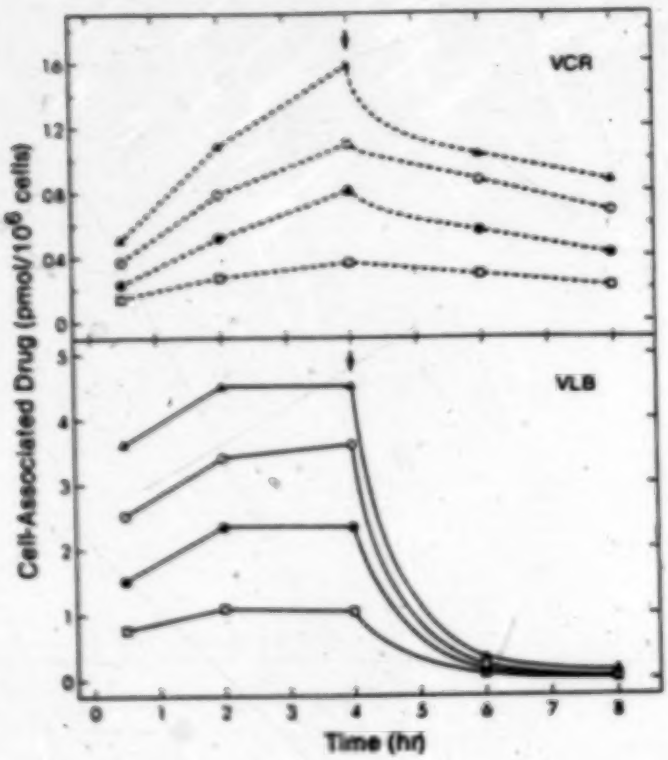

Figure 19. Uptake and release of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells subjected to 4-hr exposures to drug (10 - 40 nM). Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in 10 (□), 20 (●), 30 (○), or 40 (▲) nM [^{3}H]VCR or [^{3}H]VLB in growth medium containing 4 mM Hepes, pH 7.4. After 4 hr (arrows), cells were collected by centrifugation (120 g, 8 min) and resuspended in drug-free growth medium (approx. 1 x 10^5 cells/ml). Incubations were performed at 37°. Cell-associated radioactivity was determined at the times indicated as described in Materials and Methods (Section G), and the values (2 determinations per condition) presented were calculated assuming that cell-associated radioactivity is equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB.

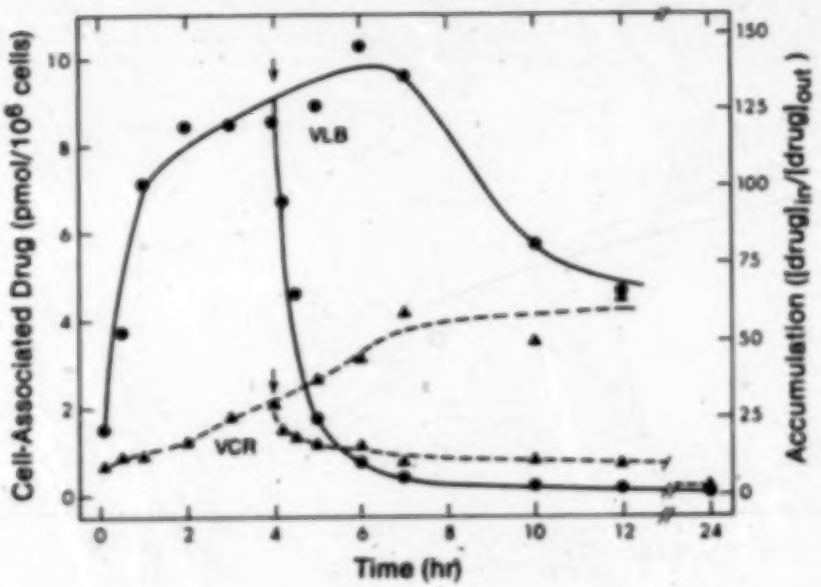

Figure 20. Time courses of uptake and release of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells subjected to 4-hr exposures to drug (50 nM). Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in 50 nM [^{3}H]VCR or [^{3}H]VLB in growth medium containing 4 mM Hepes, pH 7.4. Cultures were incubated at 37° either continuously for 12 hr or for 4 hr (arrows), followed by culture in drug-free medium for 20 hr. For the latter, cells were collected by centrifugation (120 g, 8 min) and resuspended at 1 x 10^5 cells/ml. Cell-associated radioactivity was determined at the times indicated as described in Materials and Methods (Section G), and the values (2 determinations per condition) presented were calculated assuming that cell-associated radioactivity is equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB.

crossed, and, thereafter, the amount of cell-associated VCR was greater than that of VLB.

Although cell-associated VCR continued to increase for 12 hr in the experiments of Figure 20, cell-associated VLB declined after about 7 hr. The integrity of cells after 12 and 24-hr exposures to drug was assessed by exclusion of trypan blue. At 12 hr, the percentages of dye-excluding cells were 94% (untreated cultures), 90% (VCR-exposed cultures), and 83% (VLB-exposed cultures). Cells exposed to either drug continuously for 24 hr were leaky, since only 42-46% excluded trypan blue, and thus cell-associated drug was not reported at 24 hr.

The loss of VLB from cells after prolonged drug exposures in the experiment of Figure 20 is not understood. A similar decline was seen during prolonged exposures (>24 hr) of HL-60/C1 cells to low concentrations (6-12 nM) of VLB (Figure 13, Chapter VI). If VLB release after 7 hr of continuous exposure was due to degradation of intracellular binding sites, such sites were apparently different from those that bound VCR since VCR was not released after an exposure of at least 12 hr. The decline in cell-associated VLB may be the result of vesiculation of plasma membranes, a phenomenon described for VLB-treated lymphoblastoid cells (64). The hydrophobic nature of VLB suggests that it may partition in the lipid phase of membranes, and vesiculation of plasma membranes in VLB-damaged cells could cause a decrease in cell-associated drug.

Figure 21 demonstrates the time courses of release of [^{3}H]VCR and [^{3}H]VLB from HL-60/C1 cells that were "loaded" with radiolabelled drug by incubation for 4 hr in medium containing the same concentrations of either drug. At each of these relatively high concentrations, the amount of VLB associated with cells after 4 hr was greater than that of VCR. After transfer of cells to drug-free medium, the amounts of cell-associated VLB declined to levels that were well below those of cell-associated VCR within 0.5 to 1.5 hr.

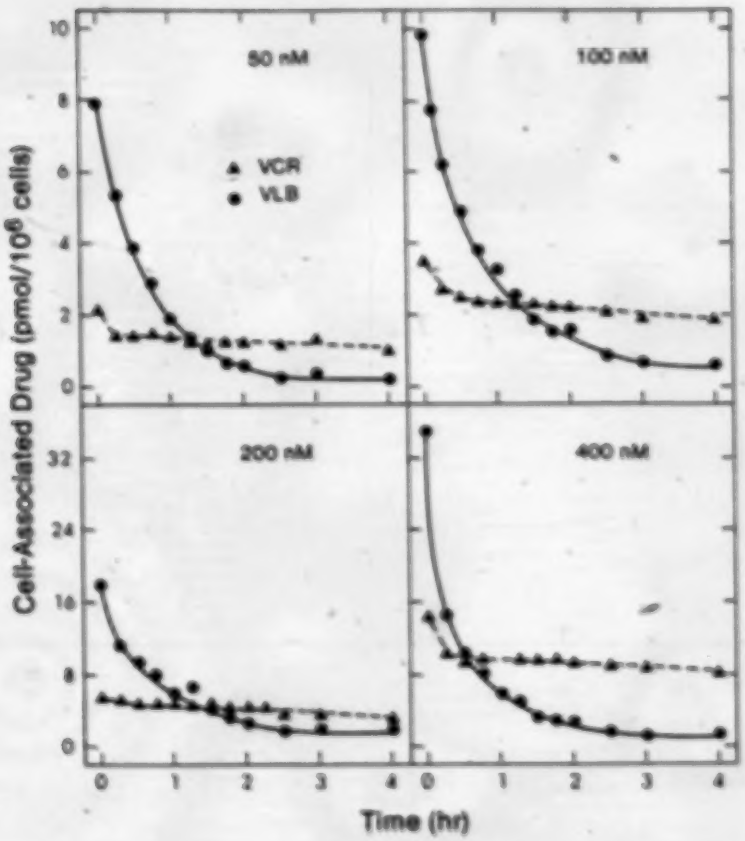

Figure 21. Time courses of release of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells after 4-hr exposures to drug (50 - 400 nM). Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in [^{3}H]VCR (▲) or [^{3}H]VLB (●) at the concentrations indicated in growth medium containing 4 mM Hepes, pH 7.4. After 4 hr, cells were collected by centrifugation (120 g, 8 min) and resuspended in drug-free growth medium (approx. 1 x 10^5 cells/ml). Incubations were performed at 37°. Cell-associated radioactivity was determined at timed intervals as described in Materials and Methods (Section G), and the values (2 determinations per condition) presented were calculated assuming that cell-associated radioactivity is equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB.

Uptake and release of [^{3}H]VLB by HL-60/C1 cells was examined at concentrations near the IC_{50} value (3.7 μM) for VLB inhibition of colony-forming ability. It is apparent from data of Figure 22 that the VLB uptake process had almost plateaued by 0.5 hr. At 4 hr, when cells were transferred to drug-free medium, VLB release was rapid; cell-associated drug had re-equilibrated with the medium by 4 hr. At 8 hr, cells were once again transferred to drug-free medium, and the amount of cell-associated VLB declined further.

Time courses of release of VCR and VLB cells were compared directly in experiments in which cells contained the same amounts of either drug (Figure 23). Cells were subjected to exposures to "pairs" of concentrations of [^{3}H]VCR and [^{3}H]VLB, respectively, that would load cells with equivalent amounts of either drug (200 and 50 nM; 400 and 100 nM). After 4 hr, loaded cells were transferred to drug-free medium, and the loss of cell-associated radioactivity was followed. For both pairs of concentrations, VLB was released more rapidly than VCR, and, after 0.5 hr, cellular contents of VCR were greater than those of VLB.

Retention of drug by cells after transfer to drug-free medium meant that exposures of intracellular target(s) continued well after exposures to extracellular drug had terminated. In addition, at the highest concentrations of VLB examined (1-6 μM), sufficient drug was released into drug-free medium that, in effect, cells continued to be exposed to low concentrations (<30 nM) of VLB. Since the cellular content of either drug was continuously changing, the "effective drug exposures" before and after transfer to drug-free medium were determined by calculating *areas under curves* of plots of *cellular drug content from time zero to infinity* . The latter was determined by extrapolation of release curves, which usually approached zero cell-associated drug by 14-24 hr. In determining "effective drug exposures", the fractions of these values that were determined from extrapolated portions of the plots of *cellular drug content versus time* were, for VCR and VLB, 25-35% and

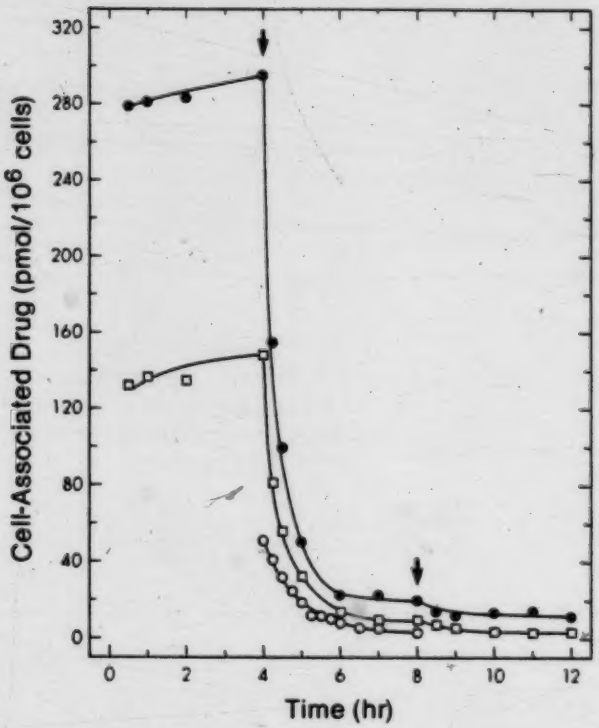

Figure 22. Time courses of release of [^{3}H]VLB by HL-60/C1 cells after 4-hr exposures to drug (1 - 6 μM). Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in 1 (○), 3 (□), or 6 (●) μM [^{3}H]VLB in growth medium containing 4 mM Hepes, pH 7.4. After 4 hr, cells were collected by centrifugation (120 g, 8 min) and resuspended in drug-free growth medium (approx. 1 x 10^5 cells/ml), and after 8 hr, cells exposed to 3 and 6 μM [^{3}H]VLB were again centrifuged (120 g, 8 min) and resuspended in drug-free medium (approx. 1 x 10^5 cells/ml). Incubations were performed at 37°. Cell-associated radioactivity was determined at the times indicated as described in Materials and Methods (Section G), and the values (2 determinations per condition) presented were calculated assuming that cell-associated radioactivity is equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB.

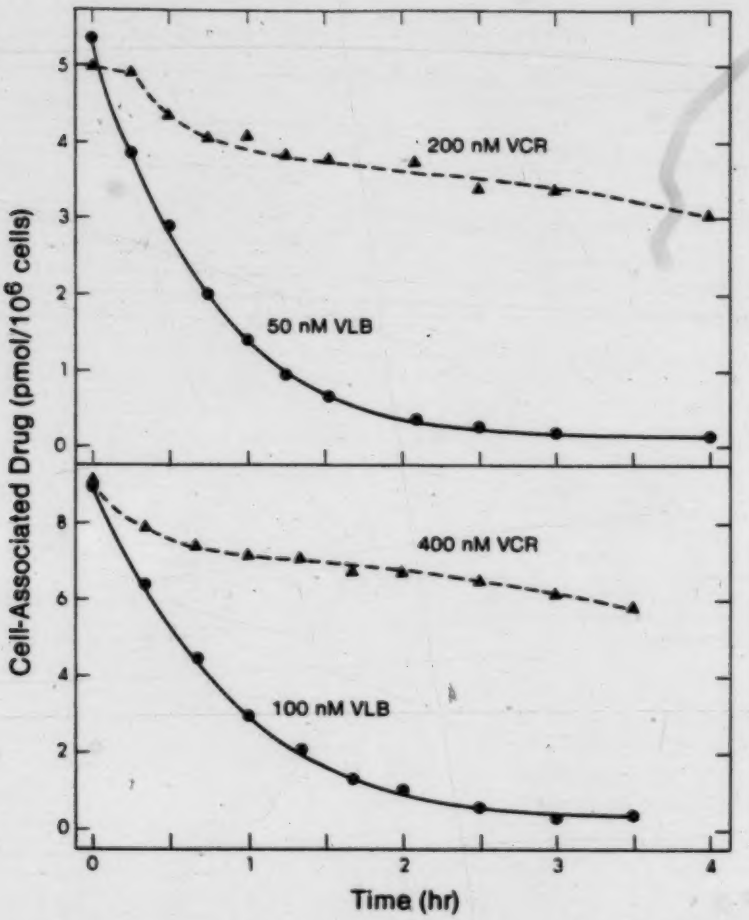

Figure 23. Time courses of release of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells containing the same amounts of either drug. Exponentially proliferating HL-60/C1 cells were used to establish cultures (1.5 x 10^5 cells/ml) in [^{3}H]VCR or [^{3}H]VLB at the concentrations indicated in growth medium containing 4 mM Hepes, pH 7.4. After 4 hr, cells were collected by centrifugation (120 g, 8 min), and resuspended in drug-free growth medium (approx. 1 x 10^5 cells/ml). Incubations were performed at 37°. Cell-associated radioactivity was determined at the times indicated as described in Materials and Methods (Section G), and the values (2 determinations per condition) presented were calculated assuming that cell-associated radioactivity is equivalent to unchanged [^{3}H]VCR or [^{3}H]VLB.

1.5-4.5%, respectively.

The relationships between "effective drug exposures" to VCR and VLB and viability of HL-60/C1 cells are presented in Figure 24. The effects of VCR and VLB on viability were determined in separate experiments (previously presented in Chapter V) by assay of colony formation under the same conditions as the uptake experiments. Maximum VCR toxicity was seen at "effective drug exposures" that were well below the threshold for VLB toxicity. These differences in sensitivities to VCR and VLB suggested that much of the cell-associated VLB was bound to cellular components other than the cytotoxic target(s).

The dependence of cellular content of [^{3}H]VCR and [^{3}H]VLB at 4 hr on the extracellular concentration of "free" drug is illustrated in Figure 25. Free drug was estimated by correcting extracellular concentrations of VCR and VLB for binding of drug to serum proteins of culture fluids.[1] Uptake of both drugs was concentration dependent, and for VLB, the plot of cell content versus free drug concentration appeared biphasic. The larger component of VLB binding did not saturate and may represent partitioning of VLB in the lipid phase of membranes. The relationships of Figure 25 were not "steady-state" values for uptake of either drug; VLB levels, which were maximal at or near 4 hr, usually declined between 4 and 8 hr, and cellular uptake of VCR did not reach maximum values until 24 hr (Chapter VI, Figure 13). It was not possible to determine if maximum cellular uptake of VCR was the same as that of VLB since, at high concentrations of VCR, cell lysis was significant during 24-hr exposures.

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[1]Depletion of drug from culture fluids did not occur in any of the experiments reported here. The maximum amount of drug accumulated by HL-60/C1 cells was $\leq$2% of total drug in culture fluids.

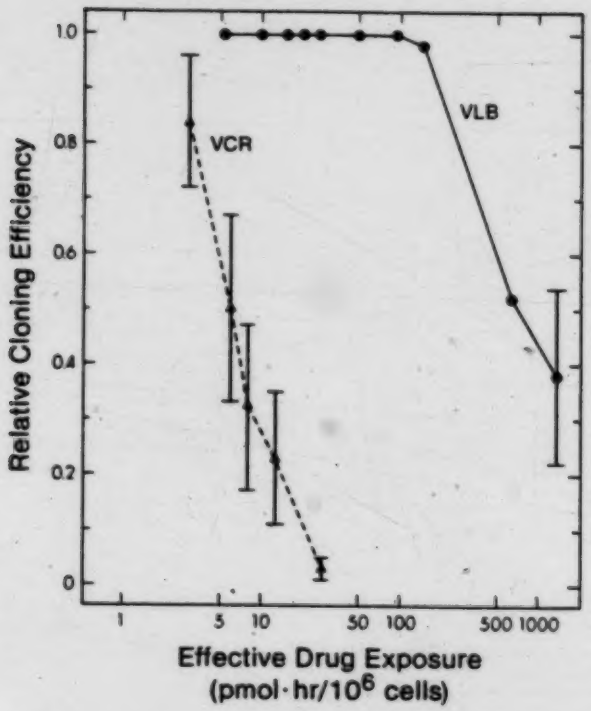

Figure 24. Colony-forming ability of HL-60/C1 cells exposed to VCR or VLB for 4 hr as a function of effective drug exposure. Uptake data are from the experiments of Figures 19-23 and viability data are from the experiments of Figure 6 (Chapter V). "Effective drug exposures" were determined from the *areas under curves* of plots of *cellular drug content from time zero to infinity*. The relative cloning efficiencies of drug-treated cultures are expressed as fractions of the absolute cloning efficiency of untreated cultures. The drug concentrations were: for VCR, 10, 20, 30, 50, and 100 nM, and for VLB, 10, 20, 30, 40, 50, 100, 200, 400, 3000, and 6000 nM. Values are presented as means (± S.D.) of relative cloning efficiencies of multiple experiments (n = 2 to 4) or of a single experiment (10 determinations per condition).

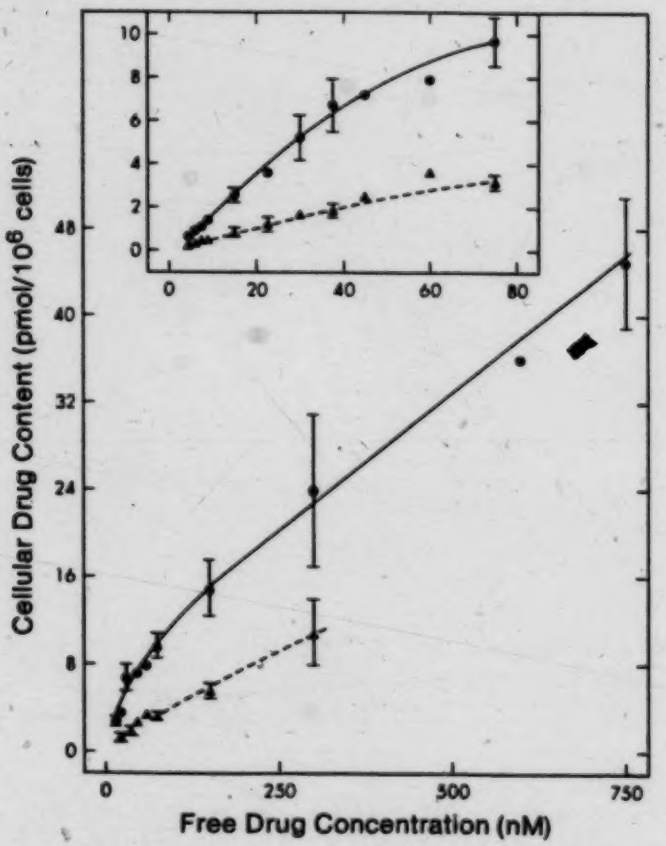

Figure 25. Cellular drug content following 4-hr exposures as a function of the concentration of free [^{3}H]VCR or [^{3}H]VLB. Data are from the experiments of Figures 13 and 19-23. The concentration of "free" drug was estimated from the measurements of binding of VCR (▲) and VLB (●) to serum proteins presented in Table 10. Inset represents data obtained using concentrations less than 80 nM. *Points with bars*, mean ± S.D.; *points without bars*, results of single determinations.

C. Summary

During 4-hr exposures, uptake of [^{3}H]VLB by HL-60/C1 cells occurred more rapidly than that of [^{3}H]VCR. Cell-associated VLB reached maximal values within 4 hr, whereas that of VCR reached maximal values only after 24 hr. When cells were exposed to the same concentrations of VCR and VLB for 4 hr, the amounts of VLB in cells were considerably greater than those of VCR. Upon transfer of cells to drug-free medium, VLB was lost from cells more rapidly than was VCR, and when cells loaded with the same amounts of drug were compared, the cellular content of VLB declined rapidly and, within 0.5 hr, was less than that of VCR.

HL-60/C1 cells were more sensitive to the cytotoxic effects of VCR, and the relationships between "effective drug exposures" and relative cloning efficiencies (viability) indicated that considerable amounts of VLB were bound to cellular components other than the cytotoxic target(s). The greater lipophilicity of VLB than VCR suggests that more VLB than VCR was loosely bound to hydrophobic components of cells, giving rise to the differences in total cell-associated drug required to achieve equivalent biological effects.

VIII. Effects of Energy Depletion on Uptake and Release of VLB

A. Introduction

Resistance of a number of cell lines to *Vinca* alkaloids has been correlated with altered membrane glycoproteins (99, 100, 103), altered intracellular calcium content (50, 95, 138, 139, 140), and efflux of drug (42, 43, 84). In resistant cells, drug efflux is inhibited by energy depletion (42, 84) and by inhibitors of Ca^{++} flux (75, 79, 89, 90, 91, 92, 97) and Ca^{++}-dependent enzyme activity (89). It is possible that the 150 kd glycoprotein seen in the plasma membranes of resistant cells is associated with an active efflux system, but this has yet to be proven. The action of inhibitors of Ca^{++} flux and calmodulin antagonists on efflux of drug from resistant cells may be manifested through perturbations of Ca^{++}-dependent processes (75, 79, 89, 90, 91, 92, 97) or by competitive or non-competitive inhibition of the efflux process (97, 98).

The basis of energy-dependent drug efflux in resistant cells is controversial. Net uptake of VCR is increased in a variety of drug-resistant cells by treatment with metabolic poisons (39, 41, 42, 43). Restoration of energy metabolism to resistant cells by addition of glucose to culture fluids stimulates drug efflux. These results have been interpreted as meaning that drug-resistant cells possess an energy-dependent transport system in the plasma membrane that mediates outward transport of drug.

A different conclusion was drawn from results of uptake studies with VLB-resistant and sensitive cultured lymphoblastoid (CCRF-CEM) cells (35). Accumulation of [^{3}H]VLB was increased by energy depletion with sodium azide in VLB-resistant cells and decreased in VLB-sensitive cells, and addition of glucose reversed these effects. VLB resistance was attributed to phosphorylation of intracellular binding sites, thereby reducing intracellular accumulation of drug. In

sensitive cells, it is possible that the putative binding sites, which may be different from cytotoxic binding sites, "sequester" drugs, thereby extending exposure of intracellular constituents to drug.

Since it seemed possible that rapid release of VLB from HL-60/C1 cells may have been dependent on an energy-dependent process, the effects of energy depletion on uptake and release of [^{3}H]VLB by HL-60/C1 cells were examined. Cells were treated with 10 mM sodium azide in the presence or absence of glucose for graded time intervals to establish conditions that depleted cells of ATP. Untreated and azide-treated cells were incubated in 50 nM [^{3}H]VLB for 4 hr and were then transferred to VLB-free medium with or without sodium azide. Measurements of cell-associated radioactivity indicated that neither uptake nor release of VLB was energy dependent. Similar experiments were not performed with VCR since an energy-dependent efflux system was not found for VLB.

B. Results

The results of Figure 26 demonstrate that, in the presence of 10 mM sodium azide and the absence of glucose, HL-60/C1 cells were completely devoid of ATP by 4 hr. In the presence of glucose, treatment of HL-60/C1 cells with sodium azide for up to 7 hr did not deplete cellular ATP, indicating that glycolysis was sufficient to meet the energy demands of HL-60/C1 cells. The chromatographic system separated adenosine mono-, di-, and triphosphates. AMP appeared in the void volume, and ADP eluted at 4, and ATP at 15, min. The additional peaks seen in extracts of untreated cells (Panel B) were other trinucleotides (CTP, GTP, and XTP).

Treatment of HL-60/C1 cells with sodium azide for extended periods affected cell integrity since recovery of cells in uptake assays declined sharply after 5-hr exposures to sodium azide in glucose-free medium (Table 12). Measurements of

Figure 26. The effects of sodium azide on the ATP content of HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (3 x 10^5 cells/ml) in growth medium (glucose-free RPMI 1640 plus 15% dialyzed FBS) containing 10 mM sodium azide with or without glucose (2 μg/ml). Untreated (control) cultures were incubated in growth medium with glucose (2 μg/ml). After the lengths of time indicated below, the variously treated cells were collected by centrifugation (120 g, 8 min), extracted with 0.4 M PCA, and prepared for HPLC analysis as described in Materials and Methods (Section H). Portions (50-55 μl) of extracts were applied to an anion exchange column and eluted as described in Materials and Methods (Section H). The absorbance (259 nM) profiles are presented for: reference standards (0.5 mM ADP, 0.1 mM ATP), Panel A; untreated (control) cells, Panel B; cells incubated in sodium azide without glucose for 0.5 hr, Panel C; cells incubated in sodium azide without glucose for 4 hr, Panel D; cells incubated in sodium azide with glucose for 4 hr, Panel E; cells incubated in sodium azide with glucose for 7 hr, Panel F.

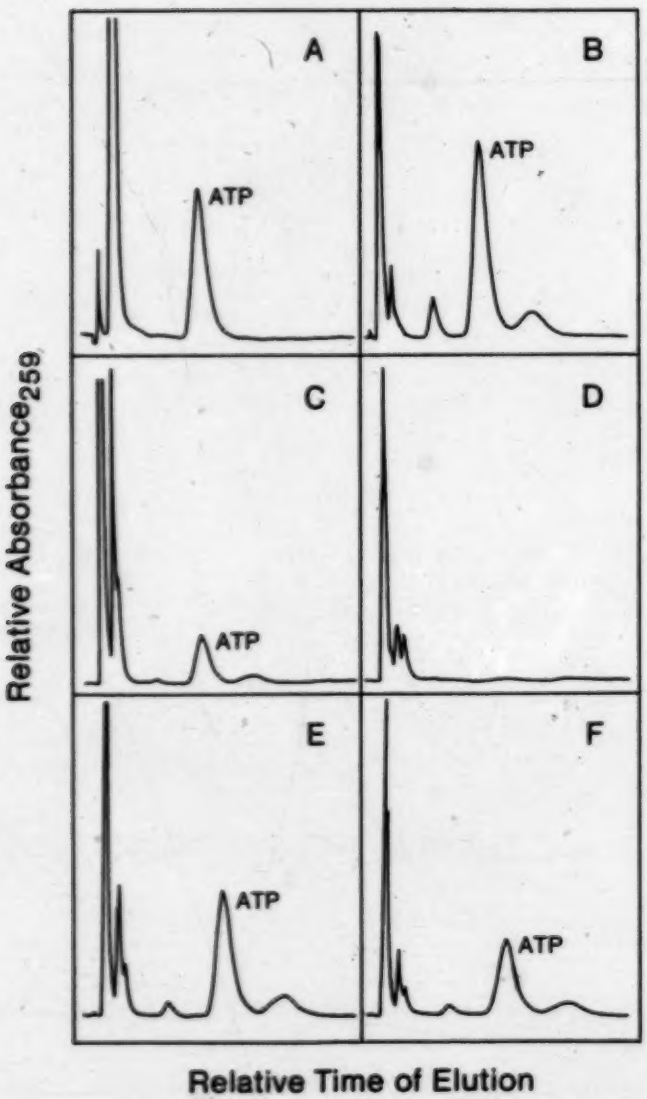
A
ATP
B
ATP
C
ATP
D
E
ATP
F
ATP
Relative Absorbance259
Relative Time of Elution

Table 12

Effects of Sodium Azide on

Cell Recovery in Uptake Assays

Time	10^{-3} x Cell Number/Pellet	
	Untreated	Azide-Treated
4	5.95	5.64
4.5	5.53	5.58
5	5.53	5.10
6	5.53	4.50
7	5.09	2.88

Exponentially proliferating HL-60/C1 cells were used to establish cultures (3×10^5/ml) in growth medium (glucose-free RPMI 1640 plus 15% dialyzed FBS) containing 50 nM VLB and either 2 μg/ml glucose (untreated) or 10 mM sodium azide (azide-treated). Cultures were incubated at 37°, and, at the times indicated, 1-ml portions of cell suspensions were processed as described in Materials and Methods (Section G) for determination of cell recovery in uptake assays. Briefly, the 1-ml samples were twice centrifuged (12,800 g, 1 min) and resuspended in 1 ml of physiological saline, and cell numbers were determined with an electronic particle counter.

cellular drug content were conducted within 4 hr of initiating treatment with sodium azide, when cell recoveries were comparable to those of untreated cells.

In the experiments of Figure 27, HL-60/C1 cells were exposed to 50 nM [^{3}H]VLB for 4 hr in the presence or absence of 10 mM sodium azide (minus glucose) and then assayed for release of radioactivity. The cellular drug contents before transfer to drug-free medium of control and ATP-depleted cells, respectively, were 5.37 and 5.07 pmol/10^6 cells indicating that ATP depletion did not effect uptake of VLB during the 4-hr incubations. These values for cell-associated VCR and VLB were equivalent to about a 75-fold "concentration" of drug. The progress curve for release of VLB from azide-treated cells was slightly steeper than that of control cells. However, the two curves were the same after 0.5 hr. Thus release of VLB from HL-60/C1 cells was not affected by depletion of cellular ATP.

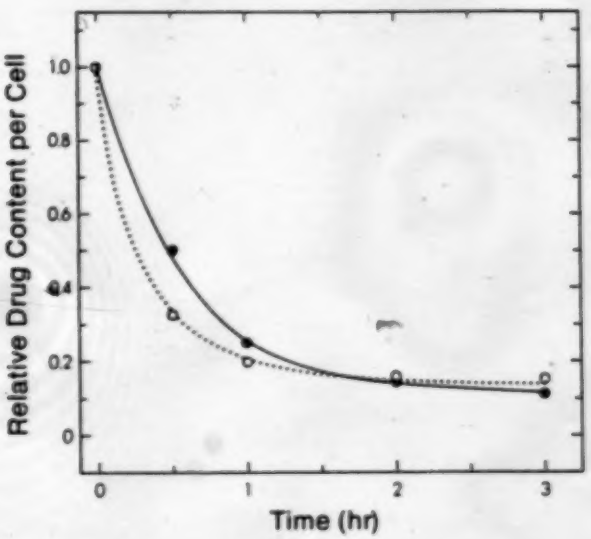

Figure 27. Effect of energy depletion on release of [^{3}H]VLB from HL-60/C1 cells. Exponentially proliferating HL-60/C1 cells were used to establish cultures (3 x 10^5 cells/ml) in growth medium (glucose-free RPMI 1640 plus 15% dialyzed FBS) containing 50 nM [^{3}H]VLB and either 2 μg/ml glucose (untreated) or 10 mM sodium azide (azide-treated). Cultures were incubated at 37°, and after 4 hr, cells were collected by centrifugation (120 g, 8 min) and resuspended in azide-free (•) or azide-containing (o) medium without [^{3}H]VLB. Cell-associated radioactivity was determined as described in Materials and Methods (Section G) and is represented as fractions of the amount of radioactivity present at 4 hr before transfer of cells to VLB-free medium.

C. Summary

Treatment of HL-60/C1 cells with 10 mM sodium azide in glucose-free growth medium reduced cellular ATP to levels below detection within 4 hr. Uptake of 50nM [^{3}H]VLB during 4-hr exposures under conditions of proliferation experiments was not affected by depletion of cellular ATP. Release of [^{3}H]VLB, after transfer of HL-60/C1 cells (loaded by incubation for 4 hr with 50 nM [^{3}H]VLB) to VLB-free medium, was the same for untreated and azide-treated cells. These results suggested that uptake and release of VLB by HL-60/C1 cells were independent of energy metabolism.

IX. Discussion

Although VCR and VLB are similar in structure (3) and bind with nearly equal affinities to *in vitro* preparations of tubulin (25), they exhibit different activities and dose-limiting toxicities in clinical use (4). The pharmacokinetics of VCR and VLB in humans are also different; VLB is distributed into body tissues and excreted more rapidly than VCR, which is retained in body tissues 3 to 4 times longer than is VLB (11, 12, 17).

A previous study established that VCR and VLB were equitoxic against 5 of 6 cell lines studied during continuous 48-hr exposures (13). Since, in patients, clearance of VCR and VLB from serum is rapid following i.v. bolus injections of VCR or VLB (11, 15, 16, 17), the effects of short exposures of cultured cells to VCR and VLB were evaluated. In 3 of 4 lines studied, VCR was much more toxic than VLB after 1 or 4-hr exposures (13).

In this work, HL-60/C1 cells were used as a model system to study the biochemical basis of differential activity of VCR and VLB. Since the accepted cytotoxic mechanism of the *Vinca* alkaloids is mitotic arrest (21, 22, 23, 24), perturbation of DNA distributions of cultured cells was assessed to determine if VCR and VLB acted by similar cytotoxic mechanisms. For both 48-hr continuous exposures and 4-hr exposures, the effects of VCR and VLB on DNA distributions were the same. At the low (<10 nM) concentrations of continuous-exposure experiments, drug-treated cells accumulated, as time progressed, in the G_2-M phases of the cell cycle. Thus, inhibition of proliferation of HL-60/C1 cells by VCR and VLB was apparently due to inhibition of progression of cells through mitosis.

When toxicities of VCR and VLB against HL-60/C1 cells were compared by assessing inhibition of proliferation during continuous exposures of 48 hr, the two drugs were equitoxic, with IC_{50} values, respectively, of 7.6 and 8.1 nM. Uptake of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells was measured over the course of 48-hr

exposures. VLB entered cells more rapidly than VCR, reaching near maximal levels within 4 hr. Accumulation of both drugs was "concentrative", and maximal accumulation was seen at or near 24 hr. The amounts of cell-associated VCR and VLB were about the same at 24 hr, suggesting, since neither drug was metabolized by HL-60/C1 cells, that cells possessed the same capacity for concentrative retention of VCR and VLB. After 24 hr, in the continued presence of extracellular drug, cell-associated VCR and VLB declined, suggesting loss of *Vinca* binding sites, possibly associated with the deteriorating condition of cells.

Since, during continuous exposures, the amounts of cell-associated VCR or VLB were constantly changing, their toxicities were compared by relating inhibition of proliferation rates to intracellular drug content over the period of evaluation. "Effective drug exposures" were determined by calculating *areas under curves* of plots of *cellular drug content versus time*, and the inhibitory effects of VCR and VLB on proliferation of HL-60/C1 cells were determined for the same exposure periods. The relationships between inhibition of proliferation and "effective drug exposures" were the same for VCR and VLB, indicating that VCR and VLB were equitoxic against HL-60/C1 cells during prolonged exposures to low concentrations of drug.

When HL-60/C1 cells were subjected to 4-hr exposures, followed by culture in drug-free medium, VCR was considerably more toxic than VLB, with IC_{50} values, respectively, of 41 nM and 1.1 μM. Uptake and release of [^{3}H]VCR and [^{3}H]VLB by HL-60/C1 cells were determined under conditions of proliferation experiments. During 4-hr exposures, uptake of [^{3}H]VLB occurred more rapidly than that of [^{3}H]VCR during the first hr, and when cells were exposed to the same concentrations of either drug, the amounts of cell-associated VLB at 4 hr were considerably greater than those of VCR. Upon transfer of cells to drug-free medium, VLB was lost more rapidly than was VCR, and when time courses of

release from cells containing the same amounts of drug were compared, cell-associated VLB was less than that of VCR within 0.5 hr.

The toxicities of VCR and VLB were compared by relating inhibition of colony formation, a measure of reproductive viability, and "effective drug exposures". The latter was determined by calculating *areas under curves* of plots of *cellular drug content from time zero to infinity*. Maximum VCR toxicity was seen at "effective drug exposures" that were well below the threshold for VLB toxicity.

That interaction of VLB with hydrophobic cellular constituents was responsible for rapid uptake and release, and the consequent low toxicity, of VLB after 4-hr exposures is suggested by the following results.

- VLB is more hydrophobic than VCR. At pH 7.4, VLB has an octanol:water partition coefficient of 2000 whereas that of VCR is 160 (38). At pH 4.9, VLB eluted in a higher concentration of methanol than did VCR in reverse phase HPLC.
- VCR and VLB have pKa values near physiological pH (38). Uptake of both drugs increased with increasing pH, suggesting that penetration of cells was more likely for neutral, than for charged, drug molecules. The pH-dependence of uptake of VLB by HL-60/C1 cells was greater than that of VCR.
- Maximum uptake of VLB by HL-60/C1 cells, which was achieved within 4 hr, increased with increasing concentrations up to 4.5 μM free drug. VLB uptake was not saturated over a wide range of concentrations.
- Although uptake of VLB was "concentrative" (intracellular levels were 100-fold greater than external concentrations), neither uptake nor release was energy-dependent.
- The cellular content of VLB after 4-hr exposures to the IC_{50} concentration (about 3 μM) was about 150 pmol/10^6 cells. Assuming that HL-60/C1 cells have similar amounts of tubulin as other human hematopoietic cell lines (79,

90), the maximum amount of VLB that could be accounted for by binding to tubulin would be of the order of 20 pmol/10^6 cells.

- At all concentrations from 6 nM to 6 μM, VLB entered HL-60/C1 cells rapidly, such that maximum levels of cell-associated VLB were reached within 0.5 to 2 hr, depending on drug concentration. Uptake of VLB was always faster than that of VCR at equal extracellular concentrations, and the cellular content of VCR reached that of VLB only if the cells were exposed for 24 hr.
- HL-60/C1 cells containing the same amounts of VCR or VLB released VLB more rapidly than VCR, independently of whether exposures were 4 or 24 hr.

VCR and VLB probably cross plasma membranes by diffusion, and, since VLB is less polar than VCR, the differences in uptake of VCR and VLB by HL-60/C1 cells may reflect differences in their permeability coefficients. The apparent non-saturability of cellular uptake of VLB suggests hydrophobic partitioning, and, if so, the equilibrium between hydrophobically bound and extracellular drug should be similar to that between hydrophobically bound and cytosolic drug. It seems likely that during 4-hr exposures, much of the cell-associated VLB was not associated with the cytotoxic target, tubulin, but with hydrophobic domains within the cell, and during the early phase of longer drug exposures, probably more VLB, than VCR, was associated with hydrophobic cellular constituents. The distribution between cellular constituents must have been time-dependent, since the toxicities of VCR and VLB ultimately approached equality during longer exposures.

The results of these studies suggest that VLB might be more active clinically if it is administered in continuous infusions or multiple low-dose injections rather than in single bolus injections. Some clinical studies support this view. Treatment of breast tumors was improved by therapy with VLB administered by infusions as compared to single bolus injections (141). Divided-dose administration of VLB, which has the effect of maintaining low levels of VLB in the body for extended periods,

resulted in improved responses in patients who were previously refractory to chemotherapy (142). Treatment of advanced breast cancer by sequential administration of ADR and VLB by continuous infusion resulted in an improved response rate (43%) compared with VLB administered as a single agent by i.v. bolus (20%) (143). However, in other studies, patients with breast cancer (144) and epithelial ovarian cancer (145) who had failed prior treatment did not respond to VLB administered by infusion. As well, only 16% of patients with refractory kidney tumors had partial responses to VLB infusion therapy (146). More work is required in establishing dosages and frequency of administration of infusion therapy to improve the therapeutic index of VLB in such protocols. Administration of VCR in infusions alters its toxicity (112). Infusion over 5 days resulted in increased serum concentrations of VCR, and although tumor response was greater when compared to single bolus injections, neurotoxicity was severe. From analysis of the pharmacokinetic data, it was suggested that a lower infusion dose may achieve the same therapeutic effects with reduced neurotoxicity. Patients with non-Hodgkin's lymphomas, previously refractory to traditional chemotherapy, were treated with a 0.5-mg bolus of VCR followed by VCR infusion at 0.25 mg/m^2/day continuously over 5 days (147). Eight of 25 patients had a partial response and one a complete response. Neurotoxicity, experienced by half of the patients, was tolerable and not greater than that found with bolus VCR treatment of a similar cumulative dose. Therapeuticity appeared to be related to maintenance of a higher serum level of drug by infusion therapy.

In summary, differential toxicity of VCR and VLB towards cultured HL-60/C1 cells was attributed to the more rapid loss of VLB than VCR when cells were subjected to short exposures to these agents, followed by culture in drug-free medium. The differences in cellular uptake and release of VLB and VCR by HL-60/C1 cells appeared to be related to the greater hydrophobicity of VLB rather

than to differences in mediated uptake or efflux of the two drugs. VLB, which is considerably more hydrophobic than VCR, should diffuse across the plasma membrane more readily and should bind nonspecifically to hydrophobic cellular components to a greater extent than VCR.

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